Development of a binding assay between the HIV-1 envelope protein (gp120) and coreceptors CCR5/CXCR4 by Surface Plasmon Resonance : Screening and optimization of viral entry inhibitors

Bridgette Janine Connell

UNIVERSITÉ DE GRENOBLE

THÈSE

Pour obtenir le grade de

DOCTEUR DE L'UNIVERSITÉ DE GRENOBLE

Spécialité : **CHIMIE ET SCIENCES DU VIVANT (218)**

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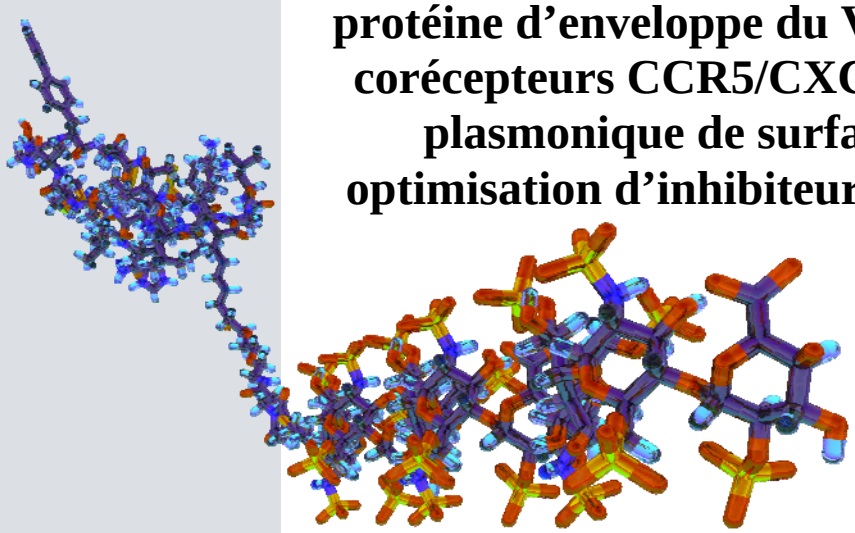
Présentée par

Bridgette Janine CONNELL

Thèse dirigée par **Hugues LORTAT-JACOB**
préparée au sein du **Laboratoire Structure and Activity of GlycosAminoGlycans (SAGAG), Institut de Biologie Structurale (IBS)**
dans **l'École Doctorale Chimie et Sciences du Vivant**

Développement d'un test d'interaction entre la protéine d'enveloppe du VIH-1 (gp120) et les corécepteurs CCR5/CXCR4 par résonance plasmonique de surface: Criblage et optimisation d'inhibiteurs de l'entrée virale



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DR, CNRS, Directeur de thèse, Examinateur
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Je remercie Prof. David Bonnaffé pour son jolie image de mCD4-HS_{12} sur la couverture ☺

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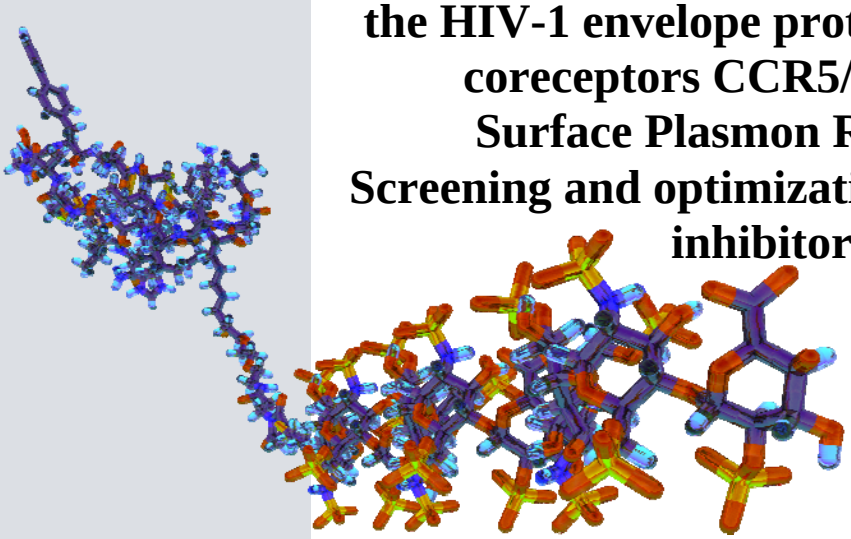

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This work is dedicated to

John Anthony CONNELL

and

George THEMISTOCLEOUS

“The tipping point is that moment when an idea, trend, or social behavior crosses a threshold, tips, and spreads like wildfire.”

— Malcolm Gladwell,

The Tipping Point: How Little Things Can Make a Big Difference

ABSTRACT

Cell-associated Heparan Sulphate (HS) binds the V3 loop of gp120 of HIV-1 thus aiding in viral infectivity. However, a soluble polyanion (HS_{12}) has anti-viral properties once conjugated to CD4 (mCD4-HS_{12}), and showed nM activity against HIV-1 *in vitro*. Due to the structural complexity of HS, screening differently sulphated-oligosaccharides to improve the molecule's activity would be too cumbersome, thus in order to obtain a more specific, higher affinity and easier to produce moiety, collaborators synthesized HS mimetic peptides. We aimed to screen these peptides and other anionic molecules for their capacity to inhibit HIV-1 entry. Thus we set-up a platform whereby solubilised CCR5 and CXCR4 were immobilized on biosensors (biacore) and used to screen for molecules that inhibited gp120-CD4 binding to the coreceptors. To control the solubilization process, CXCL12, the natural ligand of CXCR4, was injected over the immobilized CXCR4. The affinities of CXCL12 isoforms (α and γ) for CXCR4 were calculated within the ranges of previously described values with different techniques thus proving the functionality of our system. We show for the first time that HS differently regulates the binding mechanisms of these two isoforms and we propose a novel mode of action for the unusually basic C-terminal of CXCL12 γ with CXCR4. The system was subsequently used to screen the inhibitory capacity of the HS mimetic peptides. Each peptide, [S(XDXS)n], contained amino acids that mimic the hydroxyl, carboxyl and sulphate groups on HS chains. The peptide containing sulphotyrosine residues, when conjugated to mCD4 (mCD4-$P3YSO_3$), displayed nM IC_{50} for simultaneously inhibiting gp120 binding to HS, CD4, antibody, coreceptors and HIV-1 infection *in vitro*. This is the first bivalent entry inhibitor that targets both R5 and X4 viruses and the concept of a HS-mimetic peptide lends itself to structural-functional analysis of HS chains binding to proteins, a novel technique in this field.

La gp120 du VIH-1 se fixe aux héparane sulfate (HS) cellulaires, par le biais de la boucle V3 ce qui favorise l'infectivité virale. Cependant, une polyanion solubles (HS_{12}), conjugués à CD4 (mCD4-HS_{12}) a des propriétés antivirales et a montré *in vitro* une activité contre le VIH-1 à de concentrations nM. En raison de la complexité structurale des HS, le criblage d'oligosaccharides différenciellement sulfatés pour améliorer l'activité de la molécule serait trop difficile. En vue d'obtenir une molécule plus spécifique, de plus haute affinité et plus facile à produire, des peptides mimant les HS ont été synthétisés par nos collaborateurs. Notre but était de cribler ces peptides pour leur capacité à inhiber l'entrée de VIH-1. Nous avons mis en place une plateforme permettant d'immobiliser CCR5 et CXCR4 solubilisés sur des biocapteurs pour cribler des molécules qui inhibent la liaison de gp120-CD4 aux corécepteurs. Pour contrôler le processus de solubilisation, CXCL12, le ligand naturel de CXCR4, a été injecté sur CXCR4 immobilisé. Les affinités des isoformes CXCL12 (α et γ) pour CXCR4 ont été calculées dans les fourchettes de valeurs précédemment décrites avec des techniques différentes prouvant la fonctionnalité de notre système. Nous montrons pour la première fois que les HS régulent différemment les mécanismes de liaison de ces deux isoformes et nous proposons un nouveau mode d'action pour le domaine C-terminal particulièrement basique de CXCL12 γ vis-à-vis de CXCR4. Le système a ensuite été utilisé pour cribler la capacité d'inhibition des peptides mimétiques du HS. Chaque peptide, [S(XDXS)n] contient des acides aminés qui imitent les groupes hydroxyles, carboxyles et sulfates des HS. Le peptide contenant des résidus sulphotyrosines, une fois conjugué à mCD4 (mCD4-$P3YSO_3$), montre un IC_{50} de l'ordre du nM, pour l'inhibition simultanée de la liaison de gp120 aux HS, à CD4, aux anticorps, aux corécepteurs ainsi que l'infection par VIH-1 *in cellulo*. Il constitue le premier inhibiteur bivalent de l'entrée qui cible à la fois les virus R5 et X4 et le concept d'un peptide mimétique des HS se prête à une analyse structurale et fonctionnelle de la liaison des chaînes HS aux protéines, une nouvelle technique dans ce domaine.

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TABLE OF CONTENTS

ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
ARV	Antiretroviral
ART	Antiretroviral therapy
BSA	Bovine Serum Albumin
CCR5	CC Chemokine Receptor 5
CXCR4	CXC Chemokine Receptor 4
CD4	Cluster of Differentiation number 4
CD4i	CD4 induced region/domain
CDR	Complementarity-determining region
CRF	Circulating Recombinant Forms
DC	Dendritic cells
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
ECL	Extracellular loops
EMC	Extracellular Matrix
EI	Entry Inhibitor
ELISA	Enzyme linked immunosorbent assay
EMEA	European Medical Agency
Env	Envelope glycoprotein
env	Envelope gene
GAG	Glycosaminoglycans
Gal	galactose
GalNAc	N-acetyl-β-D-galactosamine
Glc	glucose
GlcA	Glucoronic Acid
GlcN	Glucosamine
GlcNAc	N-acetyl-β-D-glucosamine
Gp	Glycoprotein
GPCR	G-protein coupled receptor
HAART	Highly active antiretroviral therapy
HIV-1	Human Immunodeficiency virus type 1
HIV-2	Human Immunodeficiency virus type 2
HP	Heparin
HR-1	First heptad repeat region
HTLV	Human T-cell leukaemia virus
HR-2	Second heptad repeat region
HS	Heparan Sulphate
HSPG	Heparan Sulphate Proteoglycans
IC_{50}	Half maximal inhibitory concentration
IdoA	Iduronic Acid
IN	Integrase
k_a	Association rate constant, with units as $M^{-1}s^{-1}$
k_d	Dissociation rate constant, with units as s^{-1}
K_D	Equilibrium dissociation constant with units as M

kDa	Kilo Dalton
LAV	Lymphadenopathy-Associated Virus
LC	Langerhans cells
LTR	Long Terminal Repeat
MA	Matrix
mAbs	Monoclonal antibodies
MTCT	Mother to Child Transmission
MVB	multivesicular body
MW	Molecular weight
nM	Nanomolar
NH	Amino terminus
NMR	Nuclear Magnetic resonance
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate Buffered Saline
PCP	Pneumocystis carinii pneumonia
PCR	Polymerase chain reaction
ρg/ml	Picogram per millilitre
PHA	Phytohemagglutinin
PI	Protease Inhibitor
PIC	Pre-integration complex
PR	Protease
PSSM	Position Specific Scoring Matrices
R5	CCR5 utilizing HIV-1
RANTES	Regulated upon activation normal T-cell expressed and secreted
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute Medium
RT	Reverse transcriptase
RTI	Reverse Transcriptase Inhibitor
RT-PCR	Reverse transcription polymerase chain reaction
sCD4	Soluble CD4
SDF-1α	Stromal cell-Derived Factor – 1 alpha or CXCL12
SIV	Simian Immunodeficiency virus
SPR	Surface Plasmon Resonance
TM	Transmembrane
TPST-1	Tyrosylprotein Sulphotransferase
V3	Third variable loop
V5	Fifth variable region
wt	Wild type
X4	CXCR4 utilizing HIV-1
UNAIDS	The Joint United Nations Programme on HIV and AIDS
V	Variable Region
Vif	Viral Infectivity Factor
Vpr	Viral protein R
Vpu	Viral protein U

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A: INTRODUCTION

Chapter 1: HIV

1.1 The Global HIV/AIDS pandemic

1.1.1 Discovery and epidemiology

Acquired Immunodeficiency Syndrome (AIDS) was first detected in May 1981 among four homosexual men in Los Angeles, United States of America who presented with infections such as Pneumocystis carinii pneumonia (PCP), Kaposi's sarcoma, prolonged fever and Candida infections (1981; Gottlieb, Schroff et al. 1981). The apparent sexually transmitted immune deficiency in these patients was thought to be due to cytomegalovirus (CMV) infections in homosexual men, and called Gay-Related Immune Deficiency Syndrome (GRIDS) (1981; Gottlieb, Schroff et al. 1981; Hymes, Cheung et al. 1981; Masur, Michelis et al. 1981; Siegal, Lopez et al. 1981). However, this disease was not only seen in homosexual men; by 1983 groups of intravenous drug abusers, individuals receiving blood and blood products and heterosexual Haitians in America, presented with AIDS (1982; Harris, Small et al. 1983).

The causative agent of AIDS is a retrovirus that was first isolated from patients and demonstrated cytopathic effects on $CD4^+$ T cells, which was clearly distinct from the Human T-cell leukaemia virus (HTLV) and was thus classified as a Lymphadenopathy-Associated Virus (LAV); thus a member of the T-lymphotropic retroviruses (Barre-Sinoussi, Chermann et al. 1983; Gallo, Salahuddin et al. 1984; Gallo and Montagnier 2003). This virus is now called Human Immunodeficiency Virus (HIV), the etiologic agent of AIDS. HIV-1 crossed the species barrier from chimpanzees to humans during the early twentieth century and has since infected millions of humans. Origins of HIV-1 have thus been linked to the simian immunodeficiency virus (SIV) from the genus Lentiviruses of the family Retroviridae (Chakrabarti, Guyader et al. 1987; Desrosiers and Ringler 1989; Gao, Bailes et al. 1999; Hillis 2000). Currently, one percent of the world's population is infected with the worlds' fastest evolving pathogen, HIV-1 (Korber, Muldoon et al. 2000).

AIDS is characterized by the progressive depletion of $CD4^+$ T lymphocytes which play an important role in establishing and enhancing the cell-mediated and humoral immune response (Gottlieb, Schroff et al. 1981; Siegal, Lopez et al. 1981). When individuals suffer severe damage to their immune system, their vulnerability to opportunistic infections (OIs) and malignancies is heightened due to the loss of the individuals' ability to mount an effective immune response. Ultimately death results after many years of untreated infection (Gallo, Salahuddin et al. 1984).

As worldwide efforts to create awareness, prevention and treatment programs increase, so does the total number of people living with the virus. According to the UNAIDS report on the global epidemic in 2010, there were 2.6 million newly infected people in 2009 and 1.8 million AIDS deaths, bringing the total number of people living with HIV-1 as reported at the end of 2009 to 33.3 million (UNAIDS 2011). These figures are almost equivalent to 7,123 new infections and 4.931

deaths per day due to AIDS. The emergence of this pandemic has arguably been the most catastrophic event in medicine in the last 30 years (Figure 1.1).

While all countries are currently fighting the impact of this disease, sub-Saharan Africa, and Southern Africa in particular continue to bear the greatest burden of people infected with and affected by HIV-1. Just over 10% of the world's population inhabits sub-Saharan Africa, yet this region is home to 67.5% of people living with HIV-1 worldwide. In 2009, new infections in this region totalled more than those in all other regions of the world combined. In South Africa there are an estimated 5.6 million infected individuals which represents the largest number of individuals living with the virus in a single country.

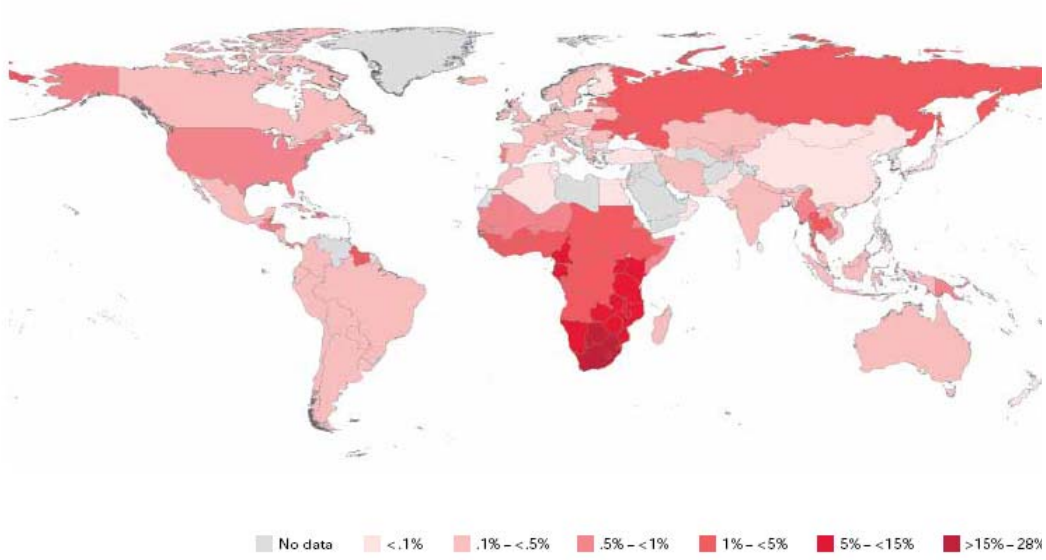


Figure 1.1Diagrammatic representation of the global prevalence of HIV infected adults and children living with HIV at the end of 2009 (UNAIDS 2011).

1.1.2 Heterogeneity

Genetic diversity of HIV-1 exists along the entire length of the genome between viral isolates from different individuals and between viral quasispecies within the same individual. The unique and unstable characteristics of HIV-1 are its inherent variability and capability of generating quasispecies as a direct result of two features; lack of a proof-reading mechanism by the viral reverse transcriptase (RT) enzyme during replication (Roberts, Bebenek et al. 1988) and its rapid replication rate (Ho, Neumann et al. 1995; Wei, Ghosh et al. 1995). The error prone RT has an estimated misincorporation (insertions/deletions) rate of 1×10^{-4} - 3.4×10^{-5} per base pair per replication cycle (Preston, Poiesz et al. 1988; Roberts, Bebenek et al. 1988; Nowak 1990; Pathak and Temin 1990; Mansky and Temin 1995; Mansky 1998). This equates to about one nucleotide being miss-incorporated per replication cycle of 9.7 kb. This process is exacerbated by the high production of approximately 1×10^{10} viral particles daily and in the absence of proof-reading mechanisms, this results in extensive viral heterogeneity (Preston, Poiesz et al. 1988; Coffin 1995; Ho, Neumann et al. 1995; Wei, Ghosh et al. 1995; Perelson, Neumann et al. 1996; Zhang, Schuler et al. 1999).

Recombination between two RNA genomes also results in major gene-rearrangements and generation of diversity within the subpopulations within the host (Jung, Maier et al. 2002; Zhuang, Jetzt et al. 2002; Levy, Aldrovandi et al. 2004). Together, these features allow HIV to rapidly mutate its genome, enabling the virus to constantly evolve and increase genetic variability. This impacts on factors such as the genotypic viral diversity amongst different isolates, immune escape and emergence of Antiretroviral (ARV) drug resistance (Mansky 1998).

1.1.3 Origins and Classification

To date, two main types of HIV have been identified with origins as zoonotic lentiviruses; HIV-1 is believed to have originated from a SIV_{CPZ} from the chimpanzee (*Pan troglodytes*) population (Gao, Bailes et al. 1999; Santiago, Rodenburg et al. 2002) and HIV-2 is believed to have originated from the SIV_{SM} sooty mangabey (*Cercocebus atys*); SIV infections originated from mangabeys and ppears to be non-pathogenic, however, SIV causes AIDS-like symptoms in the Asian rhesus macaques (Gao, Yue et al. 1992; Rambaut, Posada et al. 2004). HIV-1 and 2 are transmitted in the same fashion yet HIV-2 has a lower rate of transmission, longer asymptomatic period and lower viral load; hence it is less pathogenic (Pepin, Morgan et al. 1991; Marlink, Kanki et al. 1994). HIV-2 is endemic in West Central Africa and to a lesser extent elsewhere in the world such as Europe and the West coast of India (Rubsamen-Waigmann, Briesen et al. 1991; Babu, Saraswathi et al. 1993). However, HIV-1 predominates worldwide and has a three times higher mortality rate than HIV-2 (Whittle, Morris et al. 1994).

In addition to the two main types of HIV, further classification systems have been constructed from the copious phylogenetic data analyses of the many strains of HIV-1 and HIV-2 isolated and analyzed worldwide. There are four sub-classifications for HIV-1: groups, subtypes, sub-subtypes and circulating recombinant forms (CRFs). Of the groups, the Major group (Group M) is responsible for the current global pandemic (98% of HIV-1 infections worldwide) and the Outlier Group (Group O) and New group (Group N; consisting of non-O and non-M viruses) are less globally distributed. Groups O and N are genetically both highly divergent from group M and sparsely distributed in Cameroon and West Central Africa (Charneau, Borman et al. 1994; Mauclere, Loussert-Ajaka et al. 1997; Peeters, Gueye et al. 1997; Simon, Mauclere et al. 1998).

Group M is further subdivided up into 9 distinct subtypes, namely A, B, C, D, F, G, H, J, K wherein there are two sets of sub-subtypes A1, A2 and F1, F2 respectively (Louwagie, McCutchan et al. 1993; Robertson, Anderson et al. 2000). The emergence of Circulating Recombinant Forms (CRFs) has resulted from many recombination events between different HIV-1 viruses and already 34 CRFs have been described (Karlsson, Parsmyr et al. 1994; Casado, Thomson et al. 2005; 2007). These viruses share an identical mosaic structure in their genomes as they have descended from the same recombination events (Robertson, Anderson et al. 1999).

Phylogenetic analysis has revealed that the origin of HIV-1 came from four different cross-species transmissions from chimpanzees and one or two of these transmissions have been by gorillas (Sharp and Hahn 2010). It is generally

accepted that humans became infected by HIV-1 due to an inter-species transmission between SIV infected primates and humans. African people used to ingest simian meat that they hunted or acquired at "bushmeat markets". In this way, they were exposed to the contaminated meat.

1.1.4 Transmission

HIV-1 is transmitted through bodily fluids such as blood, semen and breast milk. Thus there are several pathways through which the virus can be transmitted between human beings; sexual transmission is the most common type of transmission. The epidemic in sub-Saharen Africa, which is responsible for almost 70% of the global infected population, is brought about (for the majaroity) by heterosexual transmission. However, in America and Europe, the epidemic is largly due to homosexual transmission. According to UNAIDS, the sharing of infected needles among injection drug users (IDU) is responsible for more than 80% of all HIV-1 infections in Eastern Europe and Central Asia. Another mode of HIV-1 transmission, also rife in sub-Saharan Africa, is the transmission from mother to child during natural child birth and during breast-feeding in conjunction with the use of formula milk.

1.1.5 Disease Pathogenesis and Progression

HIV-1 infection is characterised by a gradual deterioration in immune function and ultimately AIDS. Pathogenesis studies of HIV-1 explore the diverse mechanisms that lead to this immune system destruction and understanding how the virus establishes infection is essential to the identification and development of effective therapeutics and vaccines.

HIV-1 infection consists of an initial acute phase of infection followed by a period of clinical latency and finally a chronic phase. The acute phase is characterised by an increase in viral RNA (viral load) and the consequent decline in $CD4^+$ T cells in peripheral blood (Clark and Shaw 1993). The activation of the immune system subsequently results in the suppression of viremia to a low steady state level termed the viral setpoint, and an increase in $CD4^+$ T cells. During the clinical latency phase, viral load as well as the number of $CD4^+$ T cells may remain constant for several years with the patient remaining largely asymptomatic. However the steady replication of HIV particles eventually overwhelms the immune system, resulting in a gradual rise in viremia and a steady decrease in $CD4^+$ T cells until the patient is severely immunocompromised, resulting in increased susceptibility to opportunistic infections and the development of AIDS (Figure 1.2).

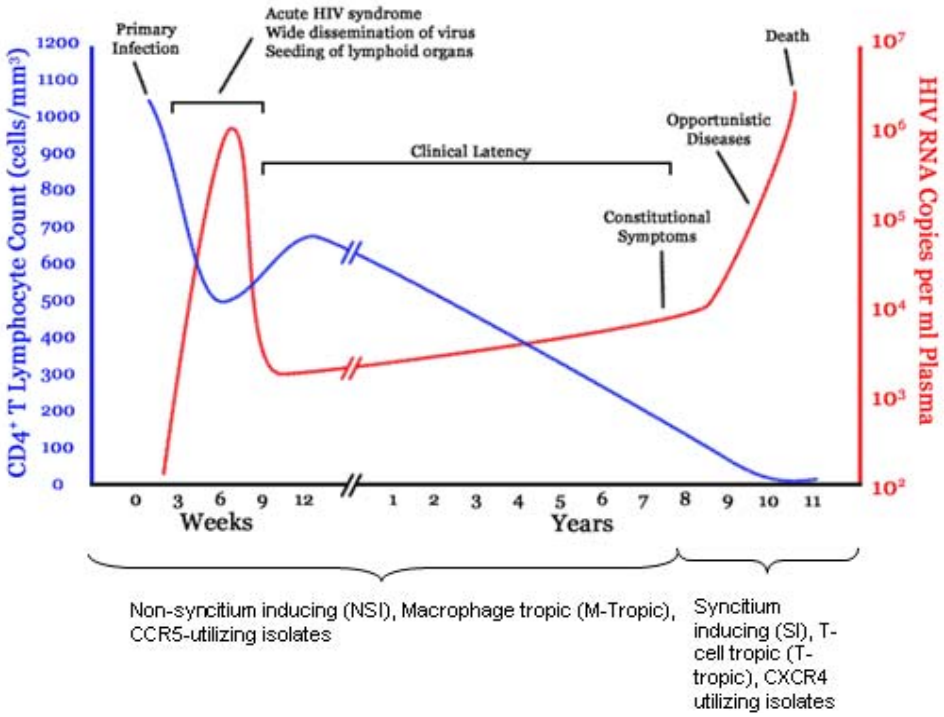


Figure 1.2 Early after primary infection there is widespread dissemination of virus and a sharp decrease in $CD4^+$ T cells count in peripheral blood. The host launches an immune response to HIV-1 characterised by a decrease in detectable viremia followed by a prolonged period of clinical latency. The $CD4^+$ T-cell count continues to decrease during the following years (in un-treated patients), until it reaches a critical level below which there is a substantial risk of opportunistic infections (Pantaleo, Graziosi et al. 1993).

1.2 The structure and Life cycle

1.2.1 Viral particle and genome

An intact, mature T-lymphotropic HIV-1 retrovirus is spherical in shape, with a diameter of approximately 80 to 120 nm (Barre-Sinoussi, Chermann et al. 1983). As in all retroviruses, HIV-1 has two copies (diploid) of identical plus-strand genomic RNA. The viral regulatory, structural and accessory proteins and enzymes are encoded by 9 partially overlapping genes spanning approximately 9.7 kb of genetic material (Figure 1.3). These 9 open reading frames code for at least 16 distinct proteins. Three of these genes encode structural proteins; Gag [group-specific antigen], Pol [polymerase] and Env [envelope]), encoded by *gag, pol and env* respectively. The Gag protein precursor is cleaved by the viral protease into the p17 matrix (MA), p24 capsid (CA), the p7 nucleocapsid protein (NC) and the P6 protein essential for viral assembly (Ganser-Pornillos, Yeager et al. 2008). The Pol protein is also cleaved by viral proteases to yield the protease (PR), reverse transcriptase (RT), RNAse and integrase (IN) enzymes which are all involved in the viral replication (Hill, Tachedjian et al. 2005).

The gp160 Envelope (Env) glycoprotein is cleaved by furin into the surface gp120 and transmembrane gp41 subunits, which are necessary for binding to the host primary receptor, CD4, and coreceptors CCR5 and CXCR4 on the surface of $CD4^+$ T cells. There are two genes that encode regulatory proteins; (Tat [transcriptional transactivator] and Rev [regulator of virion gene expression]). There are also four genes that encode accessory proteins (Vif [viral infectivity factor], Vpr [viral protein r], Vpu [viral protein u] and Nef [negative factor]) (Frankel and Young 1998; Turner and Summers 1999). The viral envelope

encases a matrix protein membrane (p17/MA), which provides further structure to the virion as well as encompasses the single layer of structural capsid/core proteins (p24/CA). The capsid contains the viral RNA, PR, RT heterodimer (comprised of two subunits; the RNAse H (p66) subunit and the RT (p51) subunit), and IN (Figure 1.3).

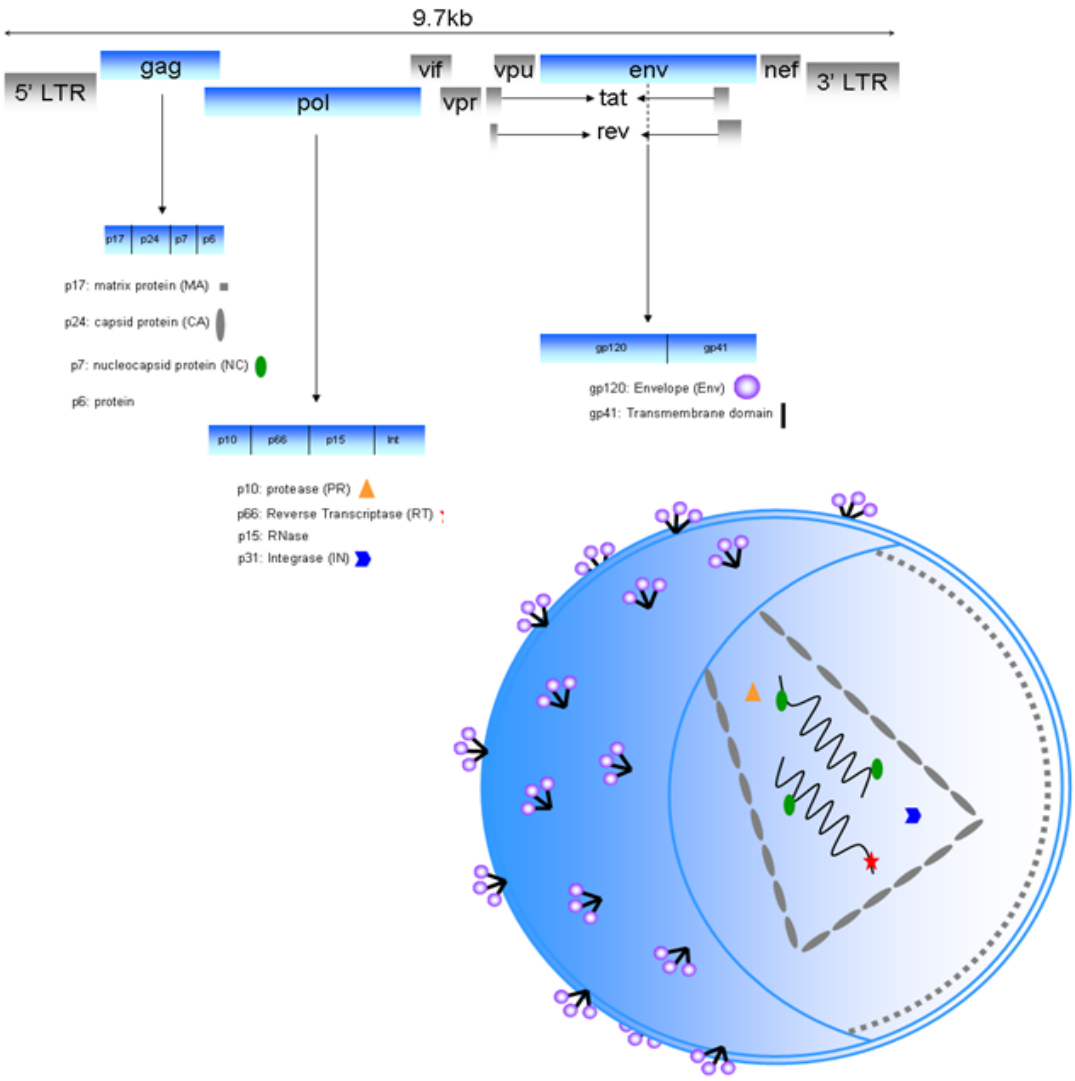


Figure 1.3 HIV structure and genome organisation: the 9 viral genes are depicted (9.7 kb) which encode open reading frames for at least 16 structural, regulatory, accessory and enzymatic proteins. The *gag*, *pol* and *env* genes encode protein precursors (pr55, pr160 and gp160 respectively) which require further processing by either viral or cellular proteases to generate structural proteins necessary for the formation of a mature virion.

1.2.2 Gp120

Viral attachment and entry into target cells is mediated by the envelope glycoprotein (env/gp160) which is initially transcribed as a non-glycosylated precursor (90 kDa).

The precursor is translated in the endoplasmic reticulum where it undergoes folding, disulphide bond formation and extensive glycosylation. About 31 oligosaccharide chains (rich in mannose) are attached to the gp120 protein at specific asparagine N-linked sites (Asn-X-Ser, Asn-X-Thr) and this glycosylation represents about 50% of the total mass of the protein (Allan, Coligan et al. 1985). The typical envelope glycoprotein has approximately 24 N-linked glycosylation sites in gp120 (Leonard, Spellman et al. 1990), as well as three or four sites in gp41 (Starcich, Hahn et al. 1986). Experimental data has revealed the presence of both complex-type, as well as high mannose or hybrid-type carbohydrates on gp120 (Leonard, Spellman et al. 1990) and these N-linked glycosylations play an essential role in neutralisation escape by HIV-1 (Figure 1.4). In this model, the coreceptor binding site is aimed directly towards the target cell membrane and is not glycosylated, and the carbohydrates shown here represent approximately half the carbohydrate on gp120, with the rest extending further from the gp120 surface (Kwong, Wyatt et al. 2000). The glycan shield protects the viral envelope from surveillance by the host immune system as the glycans are lowly immunogenic, contrary to the highly immunogenic viral proteins. This glycan shield can evolve and change rapidly, thus evading detection by the host immune system.

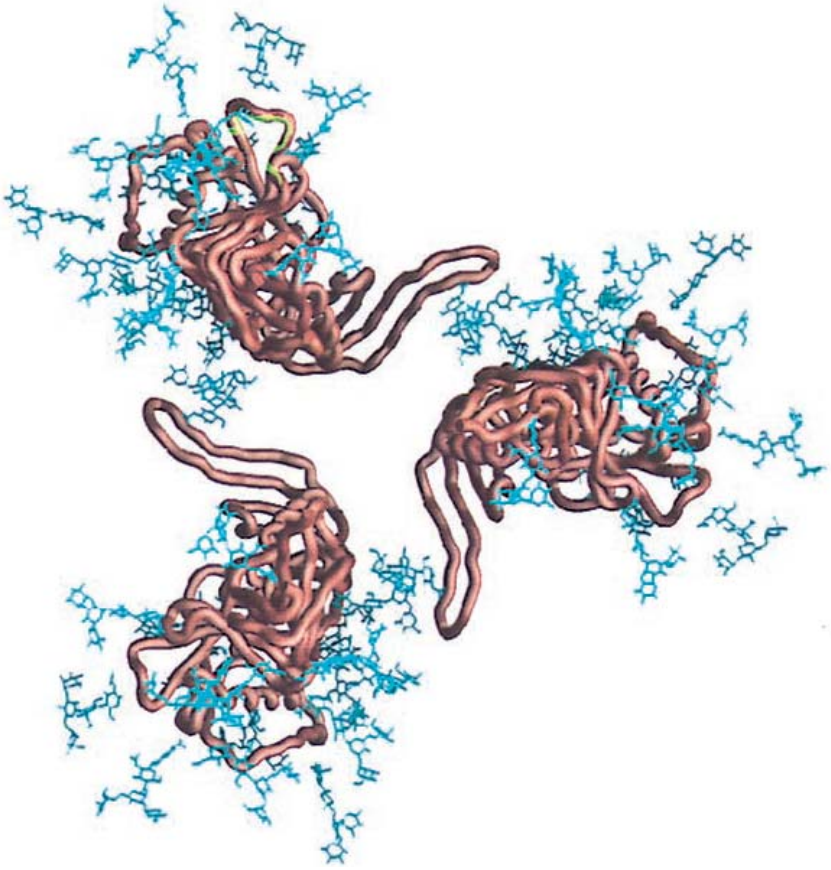


Figure 1.4 Model of gp120 trimer from the orientation of the viral membrane. The gp120 core is a copper brown and carbohydrate core structures are blue. Picture taken from (Kwong, Wyatt et al. 2000)

After glycosylation, the gp160 precursor is cleaved by convertases; furin and PC7 in the golgi apparatus into the gp120 / gp41 heterodimer, the surface and

transmembrane subunits respectively (Veronese, DeVico et al. 1985; McCune, Rabin et al. 1988; Willey, Bonifacino et al. 1988; Stein and Engleman 1990; Earl, Moss et al. 1991). The host proteases bind to a highly conserved arginine-rich cleavage site junction situated between the gp120 and gp41 peptides (Veronese, DeVico et al. 1985; Starcich, Hahn et al. 1986) (Figure 1.5 A).

Once the precursor protein is cleaved, gp120 associates non-covalently with gp41 in trimeric clusters which are transported onto the surface of budding virions (Stein, Gowda et al. 1987; Center, Leapman et al. 2002). Cryo-electron microscopy has demonstrated that despite the large variation, there are on average 14 trimeric spikes per virion which appear to be clustered (Zhu, Liu et al. 2006).

1.2.3 Structure of gp120

The gp120 envelope protein can be recognized by its five conserved regions (C1-5) and 5 variable (V1-5) regions with 18 highly conserved cysteine residues (9 disulphide bridges) (Modrow, Hahn et al. 1987; Leonard, Spellman et al. 1990). Since the advent of the structure determination of gp120, major advances have been made in understanding viral pathogenesis and the design of novel HIV entry inhibitors. Due to the extensive glycosylation of the viral envelope and the presence of poorly organized variable loops, crystallographic studies have been enormously challenging. The first crystal structure of gp120 (HXBc2) core protein was achieved by utilizing a truncated form of 120 (variable loops V1, V2 and V3 removed) in complex with domains D1 and D2 of CD4 as well as a fragment antigen binding (Fab) 17b region to stabilize the complex (Kwong, Wyatt et al. 1998). Later in 2000, the structure of a different envelope (YU2) was determined and in 2005 an HIV-1 envelope in complex with CD4 and a neutralizing antibody enabled the elucidation of the V3 loop structure (Huang, Tang et al. 2005).

HIV-1 entry requires that gp120 binds to its primary receptor CD4 and one of the family of G-coupled seven-transmembrane domain chemokine receptors, principally CXCR4 and/or CCR5 (Alkhatib, Combadiere et al. 1996; Choe, Farzan et al. 1996; Deng, Liu et al. 1996; Doranz, Rucker et al. 1996; Dragic, Litwin et al. 1996). Upon binding of CD4, gp120 undergoes drastic conformational rearrangements which causes the exposure/creation of the discontinuous coreceptor binding site or CD4 induced site (CD4i) (Dalgleish, Beverley et al. 1984; Klatzmann, Champagne et al. 1984; Maddon, Dalgleish et al. 1986; Thali, Moore et al. 1993; Kwong, Wyatt et al. 1998; Myszka, Sweet et al. 2000; Center, Leapman et al. 2002). In Peter Kwongs' first structure of the deglycosylated gp120, two major domains are revealed; an inner domain (containing the N and C terminus) and a stacked double-barrel outer domain (including the V4 and V5 loops) (Kwong, Wyatt et al. 1998). After CD4 binding, the envelope undergoes a significant re-arrangement which causes the exposure of the coreceptor binding site; the inner and outer domains of gp120 pull together in order to form the anti-paralled 4 stranded β bridging sheet (Myszka, Sweet et al. 2000). Here, the CD4 is bound at the interface between the two domains and the CD4i site flanked by the V1, V2 and V3 loops is oriented towards the cellular membrane (Figure 1.5 C) while loops V4 and V5 are situated in the outer domain. The CD4 un-bound state is also depicted, taken from Chen *et al.,* 2005 where they

solved the structure of SIV envelope glycoprotein in the absence of CD4 and thus the bridging sheet is not formed (Chen, Vogan et al. 2005) (Figure 1.5B).

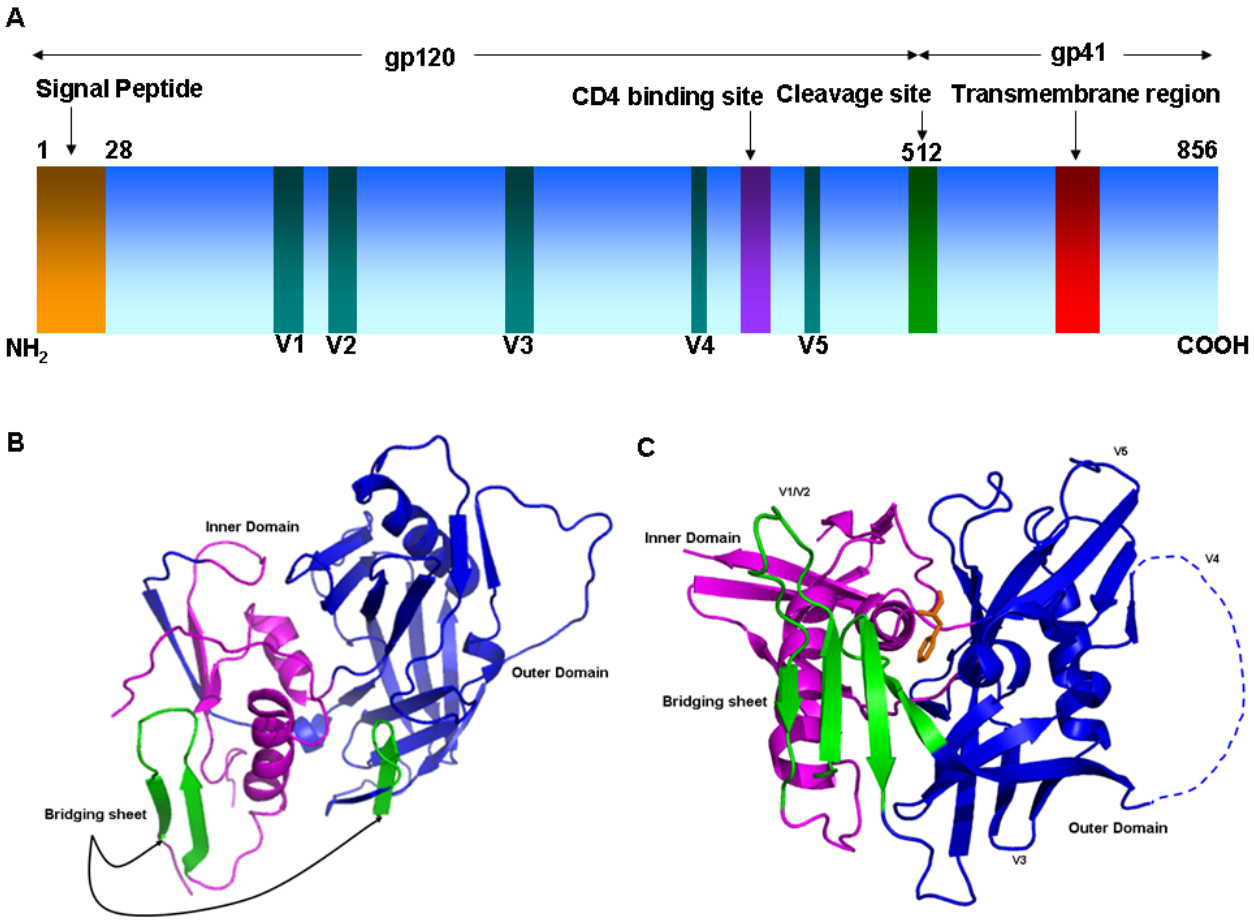


Figure 1.5 Architecture and structure of gp120. (A) The gp160 protein is cleaved by furin to produce gp120 (Env, binds to CD4 and the coreceptors) and gp41 (transmembrane fusion protein). Crystal structures of unliganded (B) and liganded (C) gp120 adapted from Kwong *et al.* (Kwong, Wyatt et al. 1998) and Chen *et al.* (Chen, Vogan et al. 2005)

Thermodynamic data has shown that gp120 undergoes a significant global re-arrangement upon CD4 binding, about 126 residues are re-organised and that in this CD4-bound state, the protein is much more stable and rigid compared to the unbound state (Myszka, Sweet et al. 2000; Zhou, Xu et al. 2007). Oligomeric modeling of gp120 suggests that the variable loops and the CD4 and neutralizing antibody binding epitopes are exposed on the gp120 before HIV-1 entry (Kwong, Wyatt et al. 2000). The model also shows that the non-glycosylated coreceptor binding domain is directed towards the host membrane with the CD4 molecules binding at angles so as not to sterically hinder each other. Thus this model proposed simultaneous binding of CD4 and the coreceptors to the trimeric gp120 heads. In order to obtain a clearer picture of how the envelope complex moves pre and post CD4 binding, Wu and colleagues have reconstructed the 3D structures of the unliganded and CD4-bound HIV-1 spikes using structural cryo-electron microscopy approaches (cryoEM), showing that the unliganded spike has a tripod

structure with distinct legs occupied by N and C termini (N/C) and the roof occupied by the variable loops. This tripod structure drastically changes when CD4 binds, as the base of the tripod becomes more dense with the variable loops

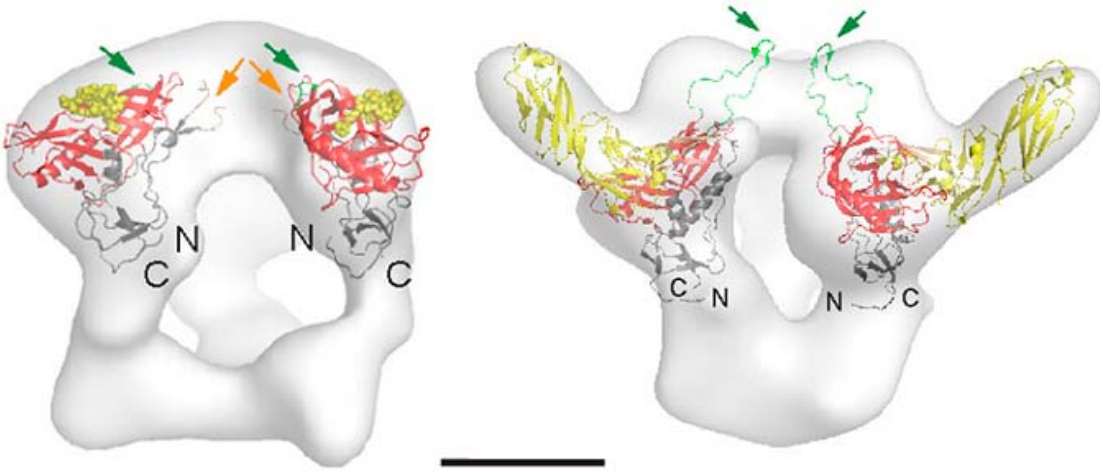


Figure 1.6 (Left) Fitting of the unliganded gp120 core (crystallographic structure) into a cryo-EM reconstruction of the unliganded HIV-1 spike. The outer and inner domains of the core gp120 are coloured red and gray, respectively, and the N/C extension blue. The stub of the V1/V2 loop is orange, whereas the stem of the V3 loop is green. The stems of the loops are additionally indicated by orange and green arrows. (Right) Fitting of the CD4-complexed gp120 core to cryo-EM reconstruction of the CD4 bound HIV-1 spike. CD4 binding loop (residues 364-374) are shown as spheres in yellow (left) and full length CD4 is in yellow on the right. Scale bar represents 50 Å. Adapted from Wu *et al.* (Wu, Loving et al. 2010)

1.2.4 The V3 Loop

In 2005, the crystal structure of gp120 in complex with CD4 and an antigen-binding fragment (Fab) of the X5 antibody (broadly neutralizing antibody directed against coreceptor-induced gp120 epitopes) was published revealing the third variable loop (V3 loop) which plays a critical role in coreceptor usage determination of HIV-1 and host cell tropism (Hwang, Boyle et al. 1991; Chan, Speck et al. 1999; Shimizu, Haraguchi et al. 1999; Hartley, Klasse et al. 2005; Huang, Tang et al. 2005). The V3 region is usually 35 amino acids in length [(31-39) amino acid positions 1 through 35 correspond to 296 through 332 in the standard reference HXBc2] with a conserved disulfide bridge at its base, a flexible stem and a β-hairpin tip. It is characteristically highly glycosylated and hypervariable however, a conserved Proline - Glycine motif on the tip of the V3 loop projects 30 Å towards to host cell membrane from the gp120 core.

This allows interaction of a four-stranded bridging sheet with the N-terminus of the CCR5 coreceptor; as the V3 tip binds to the Extracellular Cellular Loop 2 (ECL2) of CCR5 and the conserved base interacts with the sulphated CCR5 N-terminus (Huang, Tang et al. 2005; Huang, Lam et al. 2007). An indication of coreceptor usage can be made by analysing the sequence of the V3 loop; the CCR5 utilizing phenotype may be predicted (in many cases, but not all) by the presence of a neutral amino acid at position 11 in the V3 loop as well as a

negatively charged amino acid at position 25 (De Jong, De Ronde et al. 1992; Fouchier, Groenink et al. 1992; Milich, Margolin et al. 1993; Fouchier, Brouwer et al. 1995; Xiao, Owen et al. 1998; Hoffman, Seillier-Moiseiwitsch et al. 2002). Conversely, if a basic amino acid is found at position 11 and/or 24 and 25, this overall positive charge will strongly correlate with CXCR4 utilizing phenotype that binds to the negatively charged residues in the CXCR4 ECLs, however the absence of these positive residues does not rule out CXCR4 usage (De Jong, De Ronde et al. 1992; Fouchier, Groenink et al. 1992; Shioda, Oka et al. 1994; Fouchier, Brouwer et al. 1995; Milich, Margolin et al. 1997; Hoffman, Stephens et al. 1998; Brumme, Dong et al. 2004; Brumme, Goodrich et al. 2005).

There are two potential N-linked glycosylation sites within the HIV-1 subtype B V3 loop reference sequence; one appears at the N-terminal near the base of the loop (N-linked glycosylation site) and the other is found from position 6-8 (NNT). The latter, has been implicated in CCR5 usage and decreasing sensitivity to antibody neutralization (Back, Smit et al. 1994; Schonning, Jansson et al. 1996). The loss of this N-linked glycan is associated with less frequent use of the CCR5 coreceptor and in some cases, lack of the glycan is associated with exclusive CXCR4 usage (Ogert, Lee et al. 2001; Polzer, Dittmar et al. 2002).

During the course of HIV-1 infection, the transmitted virions initially enter target cells through the usage of the CCR5 coreceptor (Schuitemaker, Koot et al. 1992; van't Wout, Kootstra et al. 1994), however these virions may acquire the ability to use CXCR4 to enter the host cells (Karlsson, Parsmyr et al. 1994; Connor, Sheridan et al. 1997; Huang, Eshleman et al. 2007) – this change in tropism is generally associated with a faster progression towards AIDS. This switch in coreceptor use can sometimes be attributed to mutations within the V3 loop (Hartley, Klasse et al. 2005). Although mechanistically and structurally it is generally unclear how CXCR4 tropism is determined, the acquisition of positively charged residues in the V3 stem and perhaps other Env domains such as the V1 /V2 and gp41 may also contribute to coreceptor switching (Groenink, Fouchier et al. 1993; Koito, Harrowe et al. 1994; Koito, Stamatatos et al. 1995; Carrillo and Ratner 1996; Ross and Cullen 1998; Ogert, Lee et al. 2001; Pastore, Nedellec et al. 2006; Huang, Toma et al. 2008).

1.2.5 CD4

Cluster of differentiation 4 (CD4) is a transmembrane glycoprotein (59 kDa) and belongs to the immunoglobulin superfamily (Maddon, Littman et al. 1985). CD4 is expressed on T helper lymphocytes, macrophages, monocytes, langerhans cells, dendritic cells and microglial cells. CD4 consists of an extracellular region, a transmembrane region and a cytoplasmic region which is associated with a tyrosine kinase $p56^{Lck}$. The extracellular region of CD4 is composed of four immunoglobulin domains (D_1-D_4). D_1 and D_3 are variable immunoglobulin domains (IgV) and D_2 and D_4 are constant immunoglobulin domains (IgC). All the domains, exept for D_3, posses disulphide bridges at their bases and D_3 and D_4 posses N-linked glycosylations (Maddon, Littman et al. 1985; Clark, Jefferies et al. 1987; Maddon, Molineaux et al. 1987). CD4 on the surface of T helper lymphocytes, interacts with the β_2-domain of MHC class II molecules via its D_1 domain and increases the avidity between the T lymphocyte and the antigen-

presenting cell. In addition, the IL-16 chemokine binds to CD4 which plays a role in T-cell chemotraction (Cruikshank, Kornfeld et al. 1998).

The progressive loss of circulating T-cell lymphocytes is the hallmark of immuno-deficiency marked by disease and thus the focus of much research. This is what drove the discovery of CD4 being the primary receptor for HIV-1 (Dalgleish, Beverley et al. 1984; Klatzmann, Champagne et al. 1984) and that antibodies targeting CD4 were able to block HIV-1 entry. Through site-directed mutagenesis and x-ray chrystallography, the binding site for HIV-1 was located in the D_1 domain of CD4. Most importantly, two residues of CD4, Arg59 and Phe43, form crucial interactions between gp120 and CD4. Arg59 interacts with a hydrophobic pocket (Asp368 and Val430) of gp120, while Phe43 interacts with Asp368, Glu370, Ile371, Asn425, Met426, Trp427 and Gly473 (Kwong, Wyatt et al. 1998).

1.2.6 HIV-1 Co-Receptors

Entry of HIV-1 into target cells is initiated by the interaction of gp120 and the host cell surface receptor CD4 (Dalgleish, Beverley et al. 1984; Klatzmann, Champagne et al. 1984), as mentioned previously in section 1.2.3. This causes large conformational changes in gp120, resulting in exposure of the V3 loop as well as movement of the V1/V2 loop revealing the previously masked coreceptor binding domain or CD4 induced site (CD4i) as well as gp41 epitopes (Sattentau and Moore 1991). Although numerous coreceptors have been identified *in vitro,* β-chemokine CCR5 (Alkhatib, Combadiere et al. 1996; Choe, Farzan et al. 1996; Deng, Liu et al. 1996; Doranz, Rucker et al. 1996; Dragic, Litwin et al. 1996) and α-chemokine CXCR4 receptors (Feng, Broder et al. 1996) are the main HIV-1 coreceptors. Both these coreceptors are guanine nucleotide-binding protein (G-protein)-coupled receptors [GPCRs] that regulate migration of many different cell types once bound to their chemokine protein ligands (Baggiolini 1998; Buurman, Bradley et al. 2001; Mackay 2001).

GPCRs are the largest family of cell surface signal transduction receptors in the human genome and are involved in a multitude of systems from mediation of physiological processes associated with immunity, neuronal signalling and homeostasis to regulation of cell development, maturation and death, rendering them important targets for therapeutic and pharmaceutical intervention. This is highlighted by the fact that 30% of all known marketed medicines and drugs target GPCRs (Overington, Al-Lazikani et al. 2006). In order to better understand and design inhibitors of the gp120-CD4-GPCR interaction, structural data at the atomic scale is required for these large hydrophobic GPCRs.

From the three dimensional structures of bacteriorhodopsin and rhodopsin, related GPCRs (CCR5 and CXCR4) are predicted to contain seven membrane-spanning α-helices which assume a barrel shape in the lipid bilayer as a result of the two potential extracellular disulphide linkages linking their extracellular N-terminus and intracellular C-terminus. Recently, Wu *et al.,* determined the crystal structures of several CXCR4 homodimers in complex with small molecules, confirming the tertiary structural predictions and bringing to light the atomic level detail of this

protein which is vastly significant and important for better understanding viral entry as well as drug design (Wu, Chien et al. 2010) (Figure 1.7).

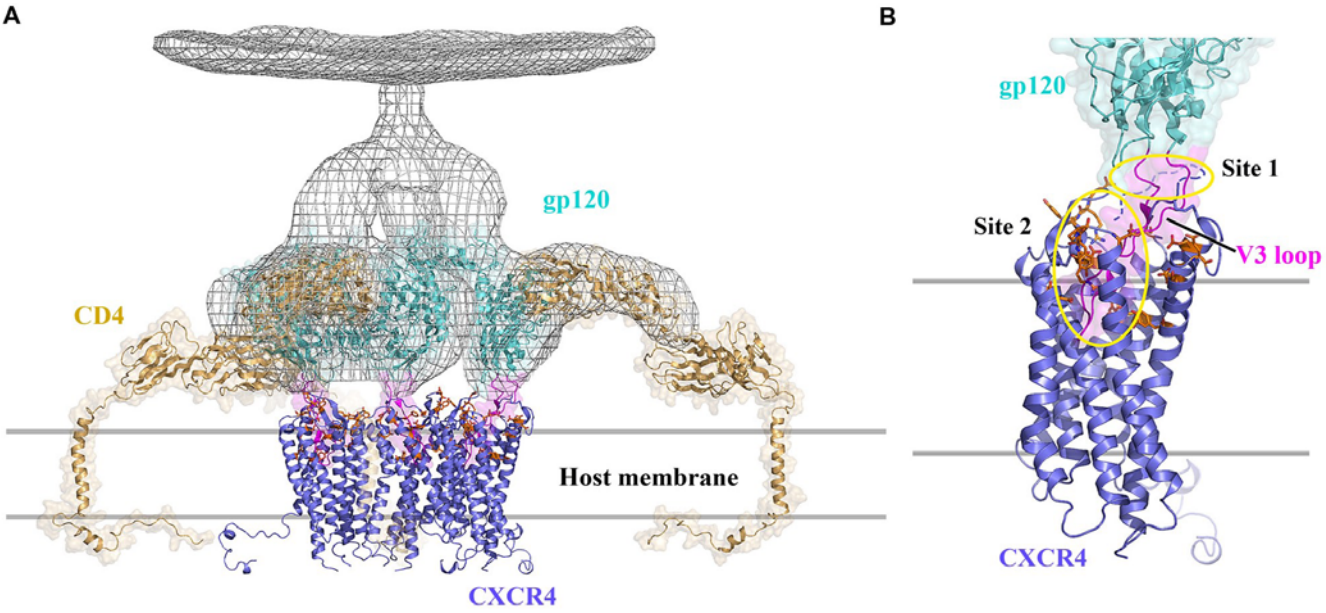


Figure 1.7 Model taken from Wu et al., (A) This is a model of the entry complex in which the crystal structure of the CXCR4 homodimer has been placed below the structures of two gp120-CD4 complexes. V3 loops are show in magenta. (B) Close-up of the V3 loop (magenta) binding to hypothetical sulphotyrosines (circled in yellow) in the N terminus of CXCR4 at site 1 which then induces further conformational changes in gp120 allowing the V3 loop to interact with ECL2 and ECL3 at site 2. CXCR4 residues that have previously been shown to participate in gp120 binding are shown in orange and the hypothetical path of the N Terminus is shown as a blue dashed line, on the left of site 1.

CCR5 undergoes *O*-linked glycosylation (Farzan, Babcock et al. 2002) and during mammalian expression, CCR5 and CXCR4 undergo *N*-linked glycosylation at one and two potential sites respectively (Helmreich and Hofmann 1996). The two potential glycosylation sites in CXCR4 (Asn11 and Asn176) were shown to be important for X4 and R5X4 viral entry. Mutation of the two N-linked glycosylation sites allows R5 viral entry through the CXCR4 (Chabot, Chen et al. 2000).

Both CCR5 and CXCR4 undergo tyrosine *O*-sulphation in the N-terminal which is thought to occur after *O*-linked glycosylation in the case of CCR5 (Mirzabekov, Bannert et al. 1999; Chabot, Chen et al. 2000). Tyrosine sulphation is a relatively widespread post-translational modification that is found in secreted, lysosymal and transmembrane proteins of multicellular organisms (Moore 2003). It has been implicated as a determinant of protein-protein interactions such as leukocyte adhesion, haemostasis and chemokine signalling (Kehoe and Bertozzi 2000). The prevalence of tyrosine sulphation is not known as there is no defined consensus sequence defined for tyrosine sulphation, however, the presence of an acidic or neutral amino acid residue directly before a tyrosine to be sulphated is correlated with tyrosine sulphation and for the case of CXCR3, CXCR4, CCR2b and CCR5. The tyrosine which is about ten amino acids on the N-Terminal side of a conserved cysteine, is *O*-sulphated (Liu, Louie et al. 2008; Stone, Chuang et al.

2009). This process occurs in the *trans*-Golgi network and is catalysed by specific sulphotransferase enzymes (Baeuerle and Huttner 1987). Up to 1% of all tyrosine residues in the total protein content of the cell can be sulphated however, the regulation of this post-translational modification is not well understood.

The modification on the tyrosine is created by the transfer of sulphate from adenosine 3' phospho-adenosine 5' phosphosulphate (PAPS) to the hydroxyl group of the tyrosine to be modified (Lee and Huttner 1983). In humans, tyrosylprotein sulfotransferase (TPST-1) and TPST-2, catalyze this reaction and TPSTs appear to be constitutively active. Both prokaryotic and eukaryotic arylsulphatases exist and reside in the lysosome and might participate in the degradation of a large array of tyrosine sulphated proteins, however, the regulation of this enzyme is not known (Parenti, Meroni et al. 1997).

Entry of CCR5 utilizing HIV-1 (R5) isolates depends largely on the amino terminus and second extracellular loop of CCR5 (Atchison, Gosling et al. 1996; Rucker, Samson et al. 1996; Doranz, Lu et al. 1997; Farzan, Choe et al. 1997) and all R5 isolates examined to date are sensitive to the loss of one or more of these sulphates. Sulphations at residues 10 and 14 in CCR5 are sufficient to facilitate interaction with HIV-1 (Cormier, Persuh et al. 2000). Not surprisingly, sulphated peptides corresponding in sequence to the CCR5 amino-terminus can slow infection of R5 isolates (Cormier, Persuh et al. 2000; Farzan, Vasilieva et al. 2000). This matter will be discussed further in Section 2.5 (Therapeutic applications of HS in HIV infection). In contrast, CXCR4 sulphation does not seem to be indispensable for the interaction of CXCR4 with HIV-1 gp120-CD4 (Lu, Berson et al. 1997; Picard, Wilkinson et al. 1997; Farzan, Babcock et al. 2002).

1.2.7 Viral Entry

Viral tropism (previously referred to as Macrophage- or T-cell tropism) is linked to coreceptor usage, with R5 viruses being M-Tropic and non syncytium-inducing (NSI) and X4 viruses being T-tropic and syncytium-inducing (SI) (Alkhatib, Combadiere et al. 1996; Choe, Farzan et al. 1996; Deng, Liu et al. 1996; Doranz, Rucker et al. 1996; Dragic, Litwin et al. 1996; Feng, Broder et al. 1996; Bjorndal, Deng et al. 1997; de Roda Husman, van Rij et al. 1999). R5 viruses are critical for HIV-1 transmission as they infect $CD4^+$ T Cells, macrophages and dendritic cells and predominate during the early stages of infection (Schuitemaker, Koot et al. 1992; van't Wout, Kootstra et al. 1994). The importance of the CCR5 coreceptor for HIV-1 transmission is emphasised by the fact that individuals bearing a homozygous 32-bp deletion in the CCR5 gene *(ccr5- Δ32)* are generally resistant to HIV-1 infection (Dean, Carrington et al. 1996; Liu, Paxton et al. 1996; Samson, Libert et al. 1996). Although R5 viruses typically persist into late disease stages, viruses that can infect $CD4^+$ T cells through binding to CXCR4, either alone (X4 viruses) or in addition to CCR5 (R5X4 viruses), emerge in approximately 50% of individuals infected with subtype B or D viruses (Karlsson, Parsmyr et al. 1994; Connor, Sheridan et al. 1997; Huang, Eshleman et al. 2007) and an increase in X4 emergence has been detected in subtype C viruses (Connell, Michler et al. 2008).

Although not required for disease progression, the appearance of X4 and/or R5X4 viruses is associated with a more rapid depletion of $CD4^+$ T cells in peripheral blood and faster progression to AIDS (Schuitemaker, Koot et al. 1992; Karlsson, Parsmyr et al. 1994; Connor, Sheridan et al. 1997; Reeves, Lee et al. 2005). However, it remains unclear whether these viruses are a cause or a consequence of accelerated $CD4^+$ T cell decline. The emergence of CXCR4-using viruses has also complicated the use of CCR5 antagonists as anti-HIV-therapeutics as these compounds can select for the outgrowth of X4 or R5X4 escape variants (Westby, Lewis et al. 2006).

HIV-1 and SIV entry into a host cell is a dynamic and complex, multi-step, cascade process. Viral entry was introduced in section 1.2.3 and here we depict the process as a three-step process. The structural intermediate (post CD4 binding and pre-coreceptor binding) is responsible for the exposure and/or formation of a chemokine coreceptor binding site, which forms the basis of the interest of the work presented here (Figure 1.8).

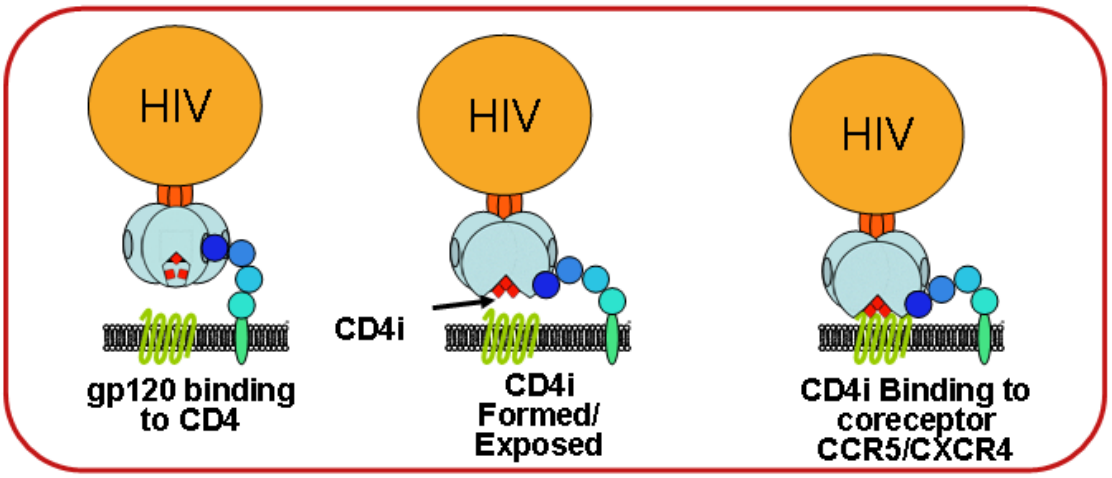


Figure 1.8 Schematic of the three classic stages of viral entry; initially the HIV-1 viral particle approaches the host cell and gp120 binds to CD4, this liaison exposes/creates the CD4i coreceptor binding domain which then permits the gp120 to recognise and bind the coreceptor CCR5 and/or CXCR4.

Actual binding of gp120 to the coreceptor (in this case CCR5) involves the V3 loop as well as the fourth constant region (C4) of gp120 (Feng, Broder et al. 1996; Trkola, Dragic et al. 1996; Wu, Gerard et al. 1996; Rizzuto, Wyatt et al. 1998; Cormier and Dragic 2002). HIV-1 neutralizing antibodies that have been raised against either the V3 loop or the C4 region are actually able to block the binding of gp120/soluble CD4 complexes to CCR5 expressing cells and prevent fusion of the virus with target cells (Feng, Broder et al. 1996; Wu, Gerard et al. 1996).

After the HIV-1 virus has bound the cell-surface CD4 and co-receptor, the gp120 protein may dissociate from the gp41 protein which is stably anchored/inserted within the viral membrane (Chen, Vogan et al. 2005). Gp41 catalyses membrane fusion. Post coreceptor binding, the gp41 fusion peptide is exposed and is harpooned and interacts with the target cell membrane, forming a pre-hairpin state that brings together the two membranes. This induces further conformational

rearrangements within the gp41, involving the antiparallel association of the two coiled heptad repeats (HR-1 and HR-2), forming a fusion active six-stranded helix bundle/hairpin core structure. This is the transition that catalyses membrane fusion. The six-helix bundle is formed before the fusion pore opening and experimental evidence suggests that fusion proceeds by lipidic intermediate states, a membrane stalk and the opening and expansion of the fusion pore (Lu, Blacklow et al. 1995; Sattentau, Zolla-Pazner et al. 1995; Chan, Fass et al. 1997; Weissenhorn, Dessen et al. 1997; Melikyan, Markosyan et al. 2000; Gallo, Finnegan et al. 2003; Pierson and Doms 2003; Buzon, Natrajan et al. 2010).

1.2.7.1 CD4-binding site and Coreceptor binding site

CD4 binding followed by coreceptor binding are the two major steps of viral entry preceeding membrane fusion. Better understanding these crucial steps from a structural and biochemical point of view will elucidate key information to designing better inhibitors of these steps. In addition, elucidation of the structure of the trimeric envelope in the different conformational states will be very beneficial for vaccine development. Liu and collegues fitted the known crystal structures of the monomeric gp120 in the unliganded and CD4-liganded conformations (Kwong, Wyatt et al. 1998; Zhou, Xu et al. 2007) into electron density maps derived by electron tomography to obtain molecular models of the HIV-1 trimer in the unliganded and CD4-bound states (Liu, Bartesaghi et al. 2008) (Figure 1.9). The viral spike contains three 'propellar-like' globular domains displaying three-fold symmetry. They demonstrate, that the CD4 binding sites on the unliganded gp120 timer are recessed about 20 Å from the top of the trimer spike with the V1/V2 and carbohydrate moieties forming a sheath at the top. Then, upon CD4 binding, each gp120 monomer rotates 45° outwards around an axis parallel to the central three-fold axis, causing a major reorganization of the gp120 trimer with an upward displacement of ~15 Å of the overall centre of mass.

Firstly the V1/V2 stem moves from the central axis of symmetry towards the lateral part of the trimer, simultaneously causing **V3 loop** movement to the distal end of the trimer directly opposite the host membrane, causing it to stick out by 30 Å above the gp120. The second coreceptor binding site is the discontinuous four antiparallel beta sheet called the **briding sheet** which is formed by the coming together of two beta sheets from each the inner and outer domains of gp120. According to the thermodynamic profiles (great magnitudes of both entropy and enthalpy) calculated for the gp120-CD4 interaction by Myszka et al., about 100 amino acids from gp120 change confirmation upon CD4 binding – this is greater than most other protein-protein binding interactions (Myszka, Sweet et al. 2000). Finally, there is also a rearrangement of the gp41 along the central axis of the trimer upon CD4 binding, most likely due to the formation of the six-helix-bundle before membrane fusion.

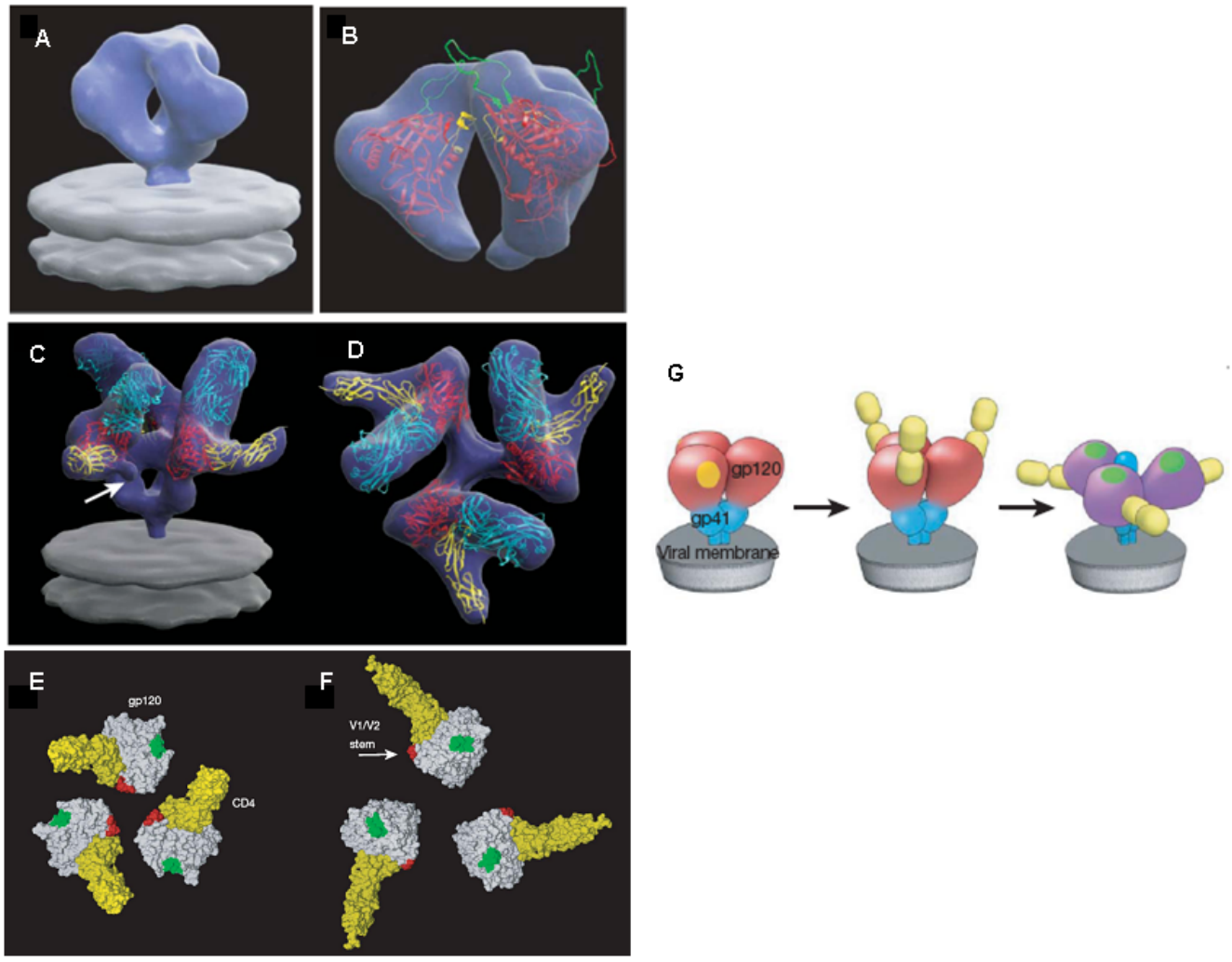


Figure 1.9 (A) Averaged three dimensional structure of the native gp120 trimeric spike surface density map. (B) Front view of the surface density map fitted with the coordinates for gp120 core (red), the V1/V2 loops (yellow) and the V3 loop (green) derived from the complex with X5 (PDB ID 2B4C). (C and D) Front and top views of the X-ray coordinates of the ternary complex of the gp120 core (red) in complex with CD4 (yellow) and Fab fragment 17b (cyan). The arrow in C points to the likely location of the V1/V2 loops. (E and F) top view showing the change from unliganded (E) to CD4-bound (F) conformational change in the gp120 trimer, gp120, CD4, V1/V2 and V3 are shown in white, yellow, red and green respectively. (G) Schematic representation showing gp41 (blue), gp120 (red/purple) regions of the trimeric spike and the conformational changes associated with CD4 (yellow) binding. The yellow spots on the gp120 show where the CD4 potentially will bind the unliganded spike and the green dots on gp120 shown the position of the V3 loops post CD4 binding (Liu, Bartesaghi et al. 2008).

1.2.8 HIV-1 Replication

The viral core is released into the cellular environment and uncoated, releasing the viral genome (Dvorin and Malim 2003). The viral RNA genome is reverse transcribed into cDNA in the cytoplasm by the viral RT (Figure 1.10) (Erikson and Erikson 1971; Sawyer, Harada et al. 1974; Marquet, Isel et al. 1995). Then the pre-integration complex (PIC) forms, consisting of an aggregation of the nascent viral cDNA, viral RT, matrix protein, integrase and Vpr. Unique nuclear localization signals (NLS) on karyophiles associated with the cell's microtubule

network direct the PIC through the host cell nuclear pores to its destination within the host nucleus (Gallay, Swingler et al. 1995; Fouchier, Meyer et al. 1998; Depienne, Mousnier et al. 2001) in both actively dividing and quiescent cells.

Viral cDNA is then irreversibly integrated into integrase-cleaved active host euchromatin to form the provirus (Sanchez-Pescador, Power et al. 1985; Bouyac-Bertoia, Dvorin et al. 2001). The provirus is flanked by the 5' LTR which serves as a promoter for transcription and the 3' LTR which provides the termination site. Phosphorylated RNA Pol II enables elongation and synthesis of full length viral transcripts (reviewed in (Jones and Peterlin 1994)). Early phase transcripts encoding the Tat, Rev and Nef proteins are spliced and are exported from the nucleus by cellular machinery. Unspliced transcripts including genomic RNA and Gag-Pol precursors as well as incompletely spliced mRNAs encoding Env, Vif, Vpr and Vpu, require the interaction between the regulatory Rev protein and the Rev responsive element present within these transcripts for nuclear export into the cytoplasm (reviewed in (Pollard and Malim 1998)).

1.2.9 Assembly, maturation and budding

Following translation, structural and enzymatic proteins collect with two copies of the viral RNA genome and assemble into immature progeny virions at the inner surface of the host cell membrane in cholesterol rich lipid rafts (Nguyen and Hildreth 2000; Liao, Cimakasky et al. 2001). Env proteins are processed into their respective subunits which also gather at the cell membrane. Nascent virions budding from the host cell results in virus particles containing trimeric Env glycoproteins embedded in host-derived membrane lipids within the viral membrane (Sakalian and Hunter 1998; Gottlinger 2001). This allows HIV to remain similar in phenotype to the host cell, contributing to the viral strategy of avoiding recognition by the host's immune system. Generally, HIV assembles at, and buds from the plasma membrane of host cells (Gelderblom 1991). However, in macrophages HIV assembles at, and buds into internal late endosomal and multivesicular body (MVB) membranes which are then transported to the cell surface and exocytosed (Orenstein, Meltzer et al. 1988; Pelchen-Matthews, Kramer et al. 2003; Pelchen-Matthews, Raposo et al. 2004). To facilitate the fission event whereby the nascent viral particle membrane is pinched off from the host membrane, a complex membrane remodelling machinery pathway is hijacked – the endosomal sorting complex required for transport (ESCRT). Gag is the principle viral protein that participates in the orchestration of HIV-1 assembly and release. At the time of viral budding, Gag associates at the inner plasma membrane, oligomerising into a type of "shell" formation, distorting the bilayer until a spherical particle buds off. It is not precisely known when, however, during or after budding, Gag is cleaved by viral proteases into the matrix protein, capsid protein and nucleocapsid proteins which are essential for viral maturation. See review (Weiss and Gottlinger 2011). Essentially, Gag hijacks the cell's machinery to cause the fission event between the viral and cellular membranes that allows for budding to occur. Maturation of progeny virions occurs following processing of the Gag and Gag-Pol polyprotein precursors extracellularly by the viral protease (Ganser-Pornillos, Yeager et al. 2008) and a single infected $CD4^+$ T cell can

produce between 10.3 x 10^9 - 10 x 10^{10} new virions per day (Ho, Neumann et al. 1995; Wei, Ghosh et al. 1995; Perelson, Neumann et al. 1996) (Figure 1.10).

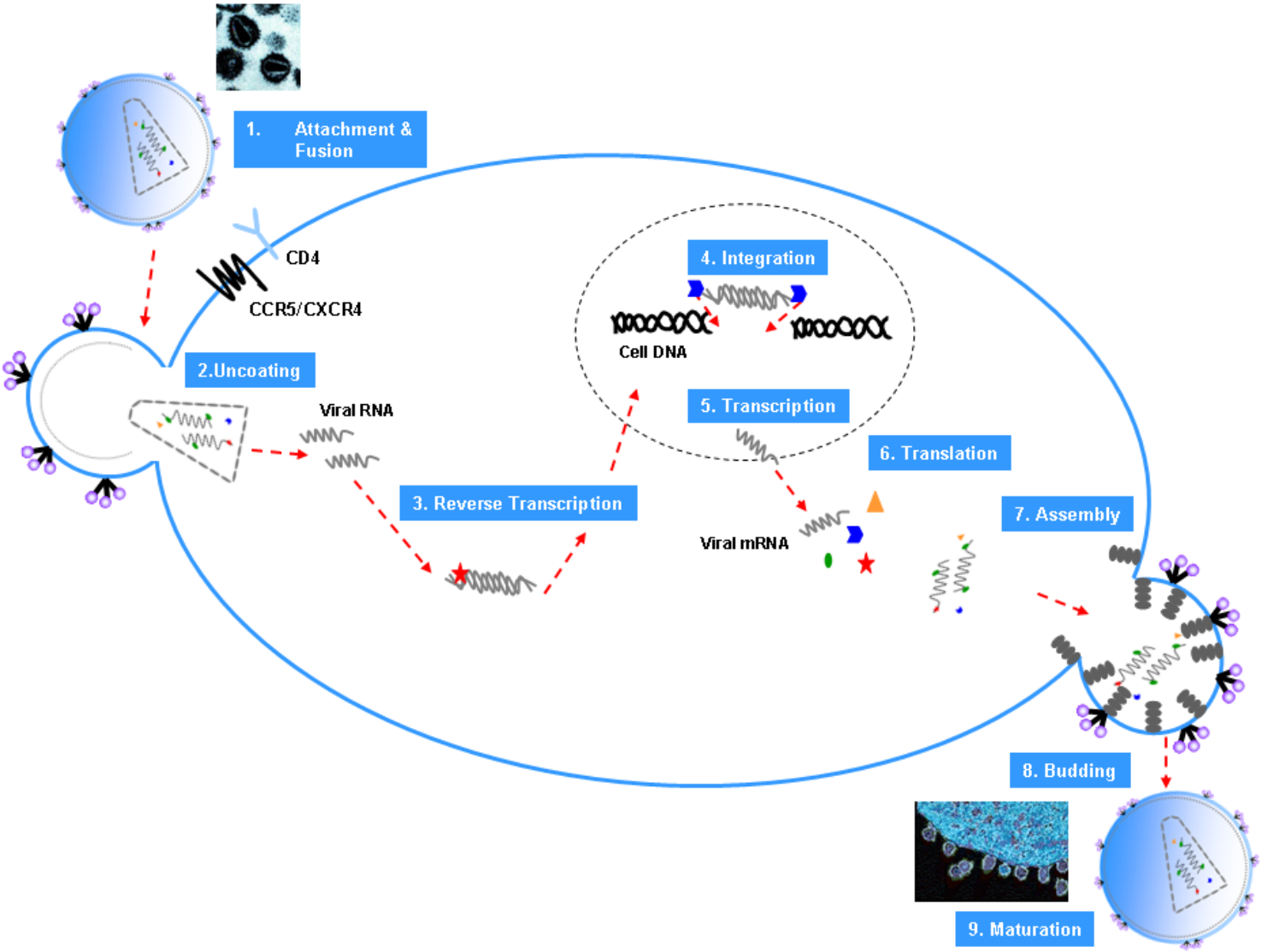


Figure 1.10 Schematic representation of the HIV-1 viral life-cycle. HIV-1 virions bind their host cell through the initial attachment to primary CD4 receptor and subsequent binding to the chemokine coreceptor CCR5 or CXCR4. Receptor binding induces fusion of viral and cellular membranes resulting in the release of the viral core and subsequent release of the viral genome into the cytoplasm of the host cell. The viral RNA genome is reverse transcribed into cDNA, transported into the nucleus where it is subsequently integrated into the host genome. The integrated provirus serves as a template for the transcription of viral genomic RNA copies as well as viral mRNA which is exported to the cytoplasm for translation. Structural and enzymatic proteins and two copies of the RNA genome assemble into nascent virion particles at the cellular membrane and bud from the cell. After their release, maturation occurs. Maturation is mediated by the protease that cleaves Gag during assembly into MA, CA, NC, SP2 an P6 proteins.

1.2.10 Host cells

HIV-1 principally binds CD4 and a GPCR to gain entry into its host cell however, other cell surface receptors also interact with gp120 and aid in the attachment / tissue invasion of the viral particles, such as lectin, DC-SIGN (Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin), glycosphyngolipides or even glycosaminoglycans (GAG), in particular heparan sulphate (HS) (Ugolini, Mondor et al. 1999).

Table 1. Cell surface receptors implicated in binding HIV virions

Receptor	Expression	Role in attachment and Infection	Reference
Gal-C	Neuronal and glial cells	Aids attachment	(Harouse, Laughlin et al. 1991)
Sulphatide (sulphate derivative of Gal-C)	Colorectal epithelial cells and primary macrophages	Confers efficient CXCR4-dependant, CD4-independent infection by NDK, a TCLA HIV-1 strain	(Fantini, Cook et al. 1993; Seddiki, Ramdani et al. 1994; Delezay, Koch et al. 1997)
Placental membrane binding protein	Cloned from placental cDNA library	Binds virus particles to the cell surface and thus enhances infectivity	(Curtis, Scharnowske et al. 1992; Geijtenbeek, Kwon et al. 2000)
DC-SIGN DC-SIGNR	Dendritic cells, endothelial cells, liver, sinusoidal and lymph node sinus endothelial cells	DC-SIGNR acts in the same way as DC-SIGN by recognizing high-mannose-containing glycoproteins	(Pohlmann, Soilleux et al. 2001)
Mannose-specific macrophage endocytosis receptor (MMR)	Macrophages	Binds gp120 and transmits the virus much like DC-SIGN does and MMR can internalize the virus	(Larkin, Childs et al. 1989)
Heparan Sulphates	Many cell types	Attaches virus particles to cell surfaces via an interaction with the V3 loop thus enhancing infectivity via CD4 and coreceptors. Acts predominantly for CXCR4-using viruses	(Mondor, Ugolini et al. 1998)
LFA-1/ICAM-1	LFA-1 is expressed on haematopoietic cells, ICAM-1 is on a wide variety of cell types	ICAM-1 incorporated onto virions which enhances attachment and infection of LFA-1+ cells	(Paquette, Fortin et al. 1998; Fortin, Barbeau et al. 1999)

1.2.10.1 DC-SIGN

Many epidemiological studies have shown that the first cells to come into contact with HIV-1 are dendritic cells (DCs) in the epidermis and mucosa (Cameron, Freudenthal et al. 1992; Weissman, Li et al. 1995). HIV-1's gp120 interacts with DCs via the DC-SIGN receptor. The dendritic cell population is highly heterogeneous and thus not all of these cells express DC-SIGN. In these cases, other lectins are expressed which have similar functions to that of DC-SIGN; e.g. the mannose receptor and langerin are expressed on the surface of Langerhans cells (Turville, Cameron et al. 2002; Nguyen and Hildreth 2003). There have been studies that suggest that langerin inhibits transmission of HIV-1 through the epithelial mucosa by endocytosing the virus into the granules of Birbeck (de Witte, Nabatov et al. 2007). The homolog of DC-SIGN, DC-SIGNR is also expressed at the surface of endothelial cells and can bind to HIV-1, in this way presenting the virus to target cells and infection occurs in *trans* (Pohlmann, Soilleux et al. 2001).

1.2.10.2 Mannose Binding Proteins (MBP)

With about half the molecular mass of gp120 attributed to N-linked carbohydrates, this is a formidable barrier for development of strong antibody responses to the virus. On the other hand, this carbohydrate barrier also provides a potential site of attack by the innate immune system through the C-type lectin mannose binding lectin (MBL) (Ji, Gewurz et al. 2005). A number of studies have shown that MBL binds to all tested HIV strains and MBL is able to inhibit DC-SIGN binding to HIV-1 (Ji, Gewurz et al. 2005). However, further studies are needed to define the *in vivo* contribution of MBL to clearance and destruction of HIV, why MBL has low neutralization of HIV-1 and if possible, how to augment anti-viral effects of MBL.

1.2.10.3 Galactosyl Ceramide (GalCer)

During early stages of infection, HIV-1 adsorbs onto the apical side of epithelial cells. The epithelial membrane has a characteristic lipid composition such that the outside layer is rich in glycosphingolipids e.g. GalCer. Galactosyl Ceramide is found on immature DCs and acts as a mucosal epithelial receptor for HIV-1, binding to gp41 (Magerus-Chatinet, Yu et al. 2007). Blocking both GalCer and CD4 with specific mAbs results in a >95% transfer inhibition of HIV-1 from human monocyte-derived DCs to autologous resting T cells (Magerus-Chatinet, Yu et al. 2007). The GalCer interaction with HIV-1 controls the early infection-independent phase of HIV-1 transfer to T cells. Thus, GalCer appears as an initial receptor for HIV-1, common to both mucosal epithelial cells and immature DCs.

1.2.10.4 Heparan Sulphates

These are complex polysaccharides present in a large quantity at the surface of most cells (see Chapter 2: for more detail). The essential property of these molecules is that they can bind a myriad of proteins, thereby altering their structure, reactivity, localization in tissues and thus have an extensive functional repertoire, see review (Whitelock and Iozzo 2005; Sarrazin, Lamanna et al. 2011). Since 1988, soluble heparan sulphates have been known to inhibit the cellular entry of enveloped viruses (Baba, Snoeck et al. 1988). In 1993, studies showed that HIV-1 interacts with heparan sulphate (HS) (Patel, Yanagishita et al. 1993; Roderiquez, Oravecz et al. 1995). Then Mondor *et al*. confirmed that HIV was

able to attach to HeLa cells via interactions between gp120 and HS (Mondor, Ugolini et al. 1998). This interaction has been demonstrated for X4 and R5X4 viruses, but is less efficient for R5 viruses (due to the number of positive charges found in the V3 loop). Since the interaction between HS and gp120 forms the base of this thesis, this subject will be discussed in further detail in Section 2.4.2.

1.2.10.5 LFA-1 / ICAM-1

Budding viruses from an infected host cell contain many host cell-surface proteins and lipids (reviewed in (Tremblay, Fortin et al. 1998)) as well as cell adhesion molecules that are thought to play a role in cell adhesion and leukocyte trafficking. Viral incorporation of foreign ICAM-1 into their membranes increases the attraction of the viral particle for the cell membrane expressing the LFA-1 integrin (Fortin, Cantin et al. 1997; Rizzuto and Sodroski 1997) and may decrease the dependence on the gp120-CD4 interaction for infection. This is confirmed by the studies that show that agents blocking the gp120-CD4 interaction are less effective at neutralizing ICAM-1 containing viruses than isogenic viruses lacking the ICAM-1 receptor (Fortin, Cantin et al. 1997; Rizzuto and Sodroski 1997).

1.3 Therapeutic Strategies

Thirty years after since the discovery of HIV-1, there is still no therapeutic cure for, nor an effective vaccine against HIV/AIDS. However, enormous efforts have been made to combat the virus. The four main classes of Food and Drug Administration (FDA) and European Medical Agency (EMEA)-approved current Antiretroviral Therapies (ARTs) for use in HIV-1 infection are; Reverse Transcriptase Inhibitors (RTIs, e.g. nucleoside/nucleotide reverse transcriptase inhibitors, NRTIs; and non-nucleoside reverse transcriptase inhibitors, NNRTIs), Protease Inhibitors (PIs), Integrase Inhibitors and Entry Inhibitors (EIs) (Fauci 2003). However, the huge burden of ART in developing countries as well as the increasing incidence of drug resistant viral strains, obliges continuous efforts for the development of new anti-HIV-1 agents. Thus the emergence of three new classes of drug targets for ART has occurred; The three classes are inhibitors targeting (i) NCp7 Zn finger inhibitors, (ii) rev/tat and (iii) viral maturation inhibitors (Huang, Maynard et al. 1998; Unwalla, Chakraborti et al. 2006; Zhou, Chen et al. 2006; Daelemans, Lu et al. 2007; Liu, Wu et al. 2007). To augment the potency of currently available ART, new approaches and more effective drugs are necessitated. Drugs targeting HIV-1 attachment *and* fusion are likely to be good targets for novel treatment strategies of HIV drug-resistant strains.

Today, there are 25 antiretroviral drugs available in 6 different classes (Zolopa 2010). These include the NNRTIs which bind directly to and inhibit RT (de Bethune 2010); entry inhibitors (CCR5 antagonists and fusion inhibitors) (Tilton and Doms 2010); and integrase inhibitors which prevent integration of the provirus into the host chromosome (McColl and Chen 2010). A novel class of antiretrovirals still undergoing clinical trial is that of the maturation inhibitors which inhibit cleavage of the capsid precursor (Temesgen and Feinberg 2006; Martin, Salzwedel et al. 2008).

1.3.1 Replication Inhibitors

The first NRTI antiretroviral drug that came out in 1987 (Ezzell 1987) inhibited the viral reverse transcriptase enzyme by acting as a nucleoside analogue, thus preventing the complete synthesis of proviral DNA (Furman and Barry 1988). However, monotherapy was not successful at repressing viral replication (Gershon 1991) and thus protease inhibitors were developed to target alternative viral components in the life cycle. These drugs inhibit viral production of mature viral proteins (Venaud, Yahi et al. 1992) and when used in combination with two NRTIs, triple therapy or highly active antiretroviral therapy (HAART) came about (Hammer, Squires et al. 1997; Hirsch, Steigbigel et al. 1999).

1.3.2 Entry Inhibition

Since gp120 has such a crucial role in HIV-1 entry, it is an attractive target for drug design, and thus a number of strategies have been aimed at disrupting the interactions between gp120 and the host receptors.

Owing to the success of entry inhibitors, an increase in the interest in the discovery and development of new molecules erupted. Logically, efforts were focused onto smaller molecules that could access the conserved and critical regions required for entry as well as be more cost-effective and easier to administer (recombinant proteins were administered through injection). For developing countries, that are most affected by the HIV epidemic, administering entry inhibitors to mucosal areas in a topical gel as a microbicide is highly suitable to the cultural and social constraints in these countries that contribute to the severity of the epidemic.

1.3.2.1 gp120-CD4 Binding Inhibitors

The idea of using soluble CD4 (sCD4) as a competitor to block the gp120-CD4 interaction was explored early in the HIV epidemic. Initially, these studies yielded promising results (Smith, Byrn et al. 1987; Deen, McDougal et al. 1988), however, sCD4 was only effective against HIV-1 laboratory-adapted strains and not primary isolates (Daar, Li et al. 1990). Seven years later a recombinant CD4-based fusion protein, Pro542 (Allaway, Davis-Bruno et al. 1995; Zhu, Olson et al. 2001) was used to block the gp120-CD4 interaction. This molecule comprises human IgG2, in which the variable domain (Fv) portions of both heavy and light chains have been replaced by the D1 and D2 domains of human CD4. Due to its tetravalent structure, Pro542 can bind Env with higher avidity than sCD4. Despite the promising results (Jacobson, Israel et al. 2004), the development of Pro542 was halted as in some cases these compounds may enhance virus entry into CCR5-expressing cells lacking CD4 (Madani, Schon et al. 2008). Recently, a functional mimetic of CD4 (M48-U1), was shown to have an EC_{50} of 25nM against SHIV162P3 due to its high affinity binding of gp120 (Van Herrewege, Morellato et al. 2008).

Small molecules that also target the CD4-binding site of gp120 are BMS-378806 and BMS-488043 and thus block CD4 binding to gp120 (Bristol Myers Squibb) (Guo, Ho et al. 2003; Lin, Blair et al. 2003; Wang, Zhang et al. 2003), however, their mode of action remains unclear (either by competing with CD4 to interact with gp120 or preventing the conformational change of gp120 required for coreceptor recognition). BMS-378806 was discontinued in Phase 1 and BMS-

488043 is in its phase IIa clinical trial. They neutralise both laboratory-adapted and primary virus isolates, including those resistant to protease and reverse transcriptase inhibitors. However, these molecules were shown to have a decreased activity against subtype C, as well as other HIV-1 subtypes when compared to its efficacy against subtype B strains (Lin, Blair et al. 2003), probably due to the inherent variability between HIV-1 envelope proteins and the use of a subtype B virus in the screening process. This drug was under investigation as a microbicide and subsequently discontinued in phase II trials (Veazey, Klasse et al. 2005), due to 40-500 fold resistance to the drug resulting from few mutations in gp120. Ibalizumab (formerly know as TNX-355) is a first-in-class, monoclonal antibody inhibitor which blocks receptor-mediated virus entry by binding to the extracellular domain 2 of the CD4. This antibody has passed the phase II trials and is mentioned in section 1.3.2.3 (Kuritzkes, Jacobson et al. 2004; Jacobson, Kuritzkes et al. 2009; Toma, Weinheimer et al. 2011).

1.3.2.2 Gp120-coreceptor binding inhibitors

A number of naturally occurring chemoattractant proteins are ligands of CCR5 or CXCR4 and thus have antiviral effects by preventing the interaction between gp120 and coreceptor and/or inducing cellular internalization of the coreceptor (Amara, Gall et al. 1997). However their clinical uses are difficult due to their intrinsic bioactivity. Derivatives of these chemokines (such as PSC-RANTES) have been engineered to reduce their agonistic effects on CCR5, and PSC-RANTES is currently being evaluated as a potential microbicide (Lederman, Veazey et al. 2004).

Blocking CCR5 is particularly attractive since the natural expression of CCR5 Δ32 homozygotes has little to no effect on patients' immune systems and general health and such individuals are highly protected against HIV-1 infection (Dean, Carrington et al. 1996; Dragic, Litwin et al. 1996; Liu, Paxton et al. 1996; Samson, Libert et al. 1996). There have been many small molecule antagonists of CCR5 blocking HIV-1 entry that have been pursued by pharmaceutical companies. These molecules are antagonists and bind within the pocket formed by the transmembrane helices (Dragic, Trkola et al. 2000; Castonguay, Weng et al. 2003; Tsamis, Gavrilov et al. 2003; Billick, Seibert et al. 2004; Nishikawa, Takashima et al. 2005; Maeda, Das et al. 2006; Seibert, Ying et al. 2006; Kondru, Zhang et al. 2008; Stupple and Ball 2011).

In August 2007, Maraviroc (UK-427857, marketed as Selzentry or Celsentry), an imidazopyridine small molecule CCR5 antagonist, was discovered through a high throughput screen of a Pfizer compound file (Dorr, Westby et al. 2005). Maraviroc demonstrated potent anti-viral activity against R5 primary viruses as well as clinically derived HIV-1 envelope-recombinant pseudoviruses, as well as viruses derived from drug-resistant patients. After positive results in clinical trials, Maraviroc was approved for use by the FDA and EMEA in 2009 (Dorr, Westby et al. 2005; Kromdijk, Huitema et al. 2010). The molecular mode of action with which Maraviroc prevents gp120 from binding to CCR5 is not clearly understood however, mutational studies and molecular modelling have shown that this small nonpeptidic ligand lodges in a hydrophobic cavity located between the transmembrane domains of the receptor, thus inhibiting gp120 binding (CCR5

unrecognisable by gp120) and chemokine signalling (MIP-1α and RANTES) by inducing conformational changes in the coreceptor (allosteric inhibition) (Dragic, Trkola et al. 2000; Tsamis, Gavrilov et al. 2003; Maeda, Das et al. 2006; Kondru, Zhang et al. 2008; Garcia-Perez, Rueda et al. 2011).

There have been other CCR5 antagonists, however, they have been less successful; TAK-779, a non-peptide compound (Baba, Nishimura et al. 1999), showed poor pharmacological and toxicological properties as well as a lack of bioavailability during clinical trails (Palani and Tagat 2006). A recent study has shown that TAK-779, like Maraviroc, also blocks HIV-1 infection through allosteric inhibition, inducing conformational changes in CCR5 thus blocking gp120 binding to CCR5 (Garcia-Perez, Rueda et al. 2011). Several other small CCR5 antagonists have demonstrated interesting efficacy against HIV. Amongst them are Aplaviroc (GW873140; GSK,) which made it to phase IIb, but was discontinued due to hepatotoxicity, and Vicriviroc (SCH-417690) is another small molecular entry inhibitor from Schering-Plough which made it to phase III trails. However, the primary efficacy endpoint was not obtained in treatment-experienced HIV-1 positive patients, thus this drug will also not be pursued (Labrecque, Metz et al. 2011).

Inhibiting the interaction between CCR5 and gp120 is successful in patients harbouring only CCR5-utilizing viruses, however, in patients with detectable levels of CXCR4-utilizing viruses, there is a strong risk that the latter population becomes dominant. The outgrowth of a CXCR4-utilizing viral population is a great concern as this is associated with accelerated $CD4^+$ T cell loss, viral load increase and disease progression (Schuitemaker, Koot et al. 1992; Karlsson, Parsmyr et al. 1994; Connor, Sheridan et al. 1997).

Hence the necessity for drugs that target the interaction between gp120 and CXCR4, however unlike CCR5, CXCR4 is essential for a myriad of normal cell functional processes, and blocking coreceptors will have negative consequences. Several peptides mimicking the natural chemokine ligand of CXCR4 (CXCL12), have been described (T-22, T-134, T-14). One such antagonist of CXCR4 is the bicyclam analogue, AMD3100 (Genzyme), which demonstrated potent activity against CXCR4-using HIV-1 *in vitro*. However, its clinical development as an antiretroviral agent was halted due to cardiac abnormalities (Dai, Yuan et al. 2010). Similarly, the development of AMD070 (Donzella, Schols et al. 1998), a third generation orally bioavailable small CXCR4 antagonist, was stopped due to liver toxicity.

1.3.2.3 Monoclonal Antibodies

Pro140 (Progenic Pharmaceuticals) is a humanized mouse monoclonal antibody directed against CCR5 that inhibits HIV-1 entry at concentrations that do not affect the chemokine receptor activity (Trkola, Ketas et al. 2001). Another monoclonal antibody that shows potential as an entry inhibitor is TNX-355 (Ibalizumab-TaiMed Biologics). TNX-355 (previously called Hu5A8) is a humanized IgG4 monoclonal antibody inhibitor that binds to the second domain of CD4 (D2) and does not prevent CD4 binding to gp120, but has shown to prevent further conformational changes in gp120 necessary for viral entry (Burkly, Olson et al. 1992; Moore, Sattentau et al. 1992). Clinical trails using this

antibody have shown some success (Jacobson, Kuritzkes et al. 2009) with weekly or biweekly dosing and phase II trials are currently underway (Toma, Weinheimer et al. 2011).

1.3.2.4 Fusions Inhibitors

Fusions inhibitors are molecules that block gp41-mediated membrane fusion. The only fusion inhibitor that has been approved by the FDA and EMEA in 2003 is a 36 mer synthetic peptide called T20 (Enfuvirtide/Fuzeon, Trimeris-Roche) which is derived from the HR2 region of gp41 (Wild, Shugars et al. 1994). T20 is able to block the formation of the six-helix bundle through its interaction with HR1, thereby preventing gp41-mediated fusion with the host membrane (Wild, Shugars et al. 1994; Kilby, Hopkins et al. 1998). This molecule is effective only after CD4 binding but prior to gp41-mediated fusion and thus has a relatively limited window during which it is active. Enfurvitide is administrated twice-daily by subcutaneous injections, however, this often results in skin sensitivity reactions at the site of injection and many patients stop treatment due to its side effects. This molecule has a relatively low genetic barrier to resistance and single amino acid mutations can lead to high levels of resistance. T-1249 is the second generation fusion peptide (Tifuvirtide) and inhibits HIV-1, HIV-2, SIV and strains that have developed resistance to T-20, however, the production of this molecule is very complex and has thus been suspended (Lalezari, Bellos et al. 2005).

Table 2: Overview of the different HIV-1 Entry Inhibitors (Castagna, Biswas et al. 2005; Kuritzkes 2009)

Entry Inhibitor	Target	Type	Administration	Clinical Trial status	Company
Dextrin-2-sulphate	Positively charged groups on surface of HIV-1	molecule	vaginal gel	Phase II	ML Laboratories
PRO 542	CD4-binding site on gp120	antibody	intravenous injection	Phase II	Progenics
TNX-355 (Ibalizumab or Hu5A8)	CD4	monoclonal antibody	intravenous injection	Phase II completed	Tanox TaiMed Biologics
BMS-488043	gp120	molecule	oral	Development discontinued	Bristol Myers Squibb
Aplaviroc (GSK-873140)	CCR5	molecule	oral	Development discontinued	GlaxoSmithKline
Maraviroc (UK 427, 857)	CCR5	molecule	oral	FDA and EMEA Approved for clinical use	Pfizer
INCB009471	CCR5	molecule	oral	Phase I/IIa completed	Incyte
HGS004	CCR5	antibody	intravenous injection	Phase I completed	Human genomic sciences
Vicriviroc (SCH417692 or SCH-D)	CCR5	molecule	oral	Phase III completed	Shering-Plough
TBR-652	CCR5	molecule	oral	Phase II completed	Tobira Therapeutics
PRO 140	CCR5	antibody	injection	Phase II	Progenics
TAK 220	CCR5	molecule	oral	Pre-clinical	Takeda
AMD 3100 (plerixafor)	CXCR4	molecule	oral	Development as antiretroviral discontinued	AnorMED Genzyme
KRH-2731	CXCR4	molecule	oral	Pre-clinical	Kureha
Enfuvirtide (T-20)	gp41	peptide	sub-cutaneous injection	FDA and EMEA Approved for clinical use	Trimeris/Roche

The two licensed entry-inhibitors are highlighted in pink

1.3.3 Neutralising Antibodies

The remarkable diversity, extensive glycosylation and conformational flexibility of the HIV-1 envelope, including the substantial rearrangement of the gp120 glycoprotein upon binding the CD4 receptor, allow it to evade antibody-mediated neutralization. Neutralizing antibodies are believed to be crucial in the protective immune response against many viral infections, yet their role in HIV-1 infection remains controversial. During classical HIV-1 infection, neutralizing antibodies appear to have little effect on acute viremia, as they arise too late and the virus readily escapes type-specific neutralizing antibodies (Richman, Wrin et al. 2003; Wei, Decker et al. 2003; Rong, Li et al. 2009). However, passive transfer of broadly neutralizing monoclonal antibodies (MAbs) has proven to be protective in nonhuman primate models (Baba, Liska et al. 2000; Mascola, Stiegler et al. 2000; Veazey, Shattock et al. 2003; Hessell, Poignard et al. 2009), supporting the hypothesis that a vaccine capable of inducing these types of antibodies is likely to be effective.

1.3.4 Vaccine and Pre-exposure Prophylaxis

Efforts to develop an effective vaccine against HIV-1 began as early as 1987, with over 80 studies making it to phase I and II trials and about 30 different candidate vaccines. However, due to the high mutation rate and replicative turnover of the virus, an effective vaccine is a difficult target to reach. Some vaccine strategies are to use inactivated or live-attenuated viruses to prime the host's immune system, however, they risk the eventual appearance of an active virus. Just over 10 years ago recombinant live-attenuated or replication-deficient viruses were investigated as vaccine platforms and have been licensed for animals. However, viral-based vaccines for humans are taking slightly longer and have to deal with the potential problem of pre-existing anti-vector immunity.

A strategy whereby viral proteins derived from the envelope were used to stimulate the production of neutralizing antibodies was recently tried (Thai Trial). This Vaccine (developed by VaxGen) entered phase III clinical trials in the U.S.A and Thailand, but has not shown a protective effect against HIV-1 infection (Desrosiers 2004; Pitisuttithum, Berman et al. 2004). Possible reasons for the failure of this vaccine are due to the heterogeneous structure of gp120 and the fact that critical coreceptor binding sites are hidden under the mass of glycans and variable loops and thus inaccessible to neutralizing antibodies.

Another strategy for vaccine design is to stimulate the host immune system (dendritic and natural killer T Cells) with viral fragments, such as "naked DNA" so that target host cells that integrate this fragment, synthesize the corresponding viral protein and present the antigen to the immune system (Nair, Heiser et al. 2000; Liao, Li et al. 2004; Melhem, Liu et al. 2007; Dell, Klein et al. 2008).

In 2009, a large phase III trial of an ALVAC and AIDSVAX vaccine (RV144) demonstrated modest protection from infection with HIV-1, with a 31% reduction among trial volunteers (Rerks-Ngarm, Pitisuttithum et al. 2009). Here, a vector-based canarypox virus and adenovirus type 5 were developed by Sanofi-Pasteur to elicit antibodies and cellular immune responses to HIV-1. A protection of 31% is

barely something to celebrate. Recently, investigators have reported the development of broadly neutralizing antibodies, which provide potential new targets for vaccine development (Wu, Yang et al. 2010; Zhou, Georgiev et al. 2010).

However, there is some hope. A private biopharmaceutical company, SEEK, is planning the final stages of development of its HIV-v vaccine, after announcing that the product has demonstrated proof of efficacy in a Phase Ib/II study. HIV-v is a T and B cell vaccine against the conserved regions (internal proteins NEF, REV, VIF and VPR) of the HIV virus and it is the first vaccine to form an antibody response against a conserved internal protein. A therapeutic trial of 55 HIV patients at 6 centres in the UK has shown a 90% reduction in the viral load of vaccinated patients compared with natural disease progression (http://www.seekacure.com/about/factsheet-HIV-v.html Access 20/07/2011).

Up until now, microbicides used to prevent vaginal transmission of HIV-1 have proved either ineffective or have even enhanced transmission in human trials (Rerks-Ngarm, Pitisuttithum et al. 2009), however, there is hope that the VOICE trail (microbicides and pre-exposure prophylaxis with ARVs targeting viral replication) will prove efficacious (Rossi 2009). Interestingly, male circumcision has provided remarkable protection from transmission, however the mechanisms are still unknown (Auvert, Taljaard et al. 2005; Bailey, Moses et al. 2007; Gray, Kigozi et al. 2007).

Results of two new studies (13 July 2011) have provided more compelling evidence that daily pre-exposure prophylaxis (PrEP) treatment with ARTs can prevent the spread of HIV-1 infection – demonstrating for the first time that the drugs significantly reduced the risk of acquiring the AIDS-causing virus in heterosexual men and women, the population hardest hit by the disease. This is ground-breaking news and future efforts at curbing the HIV-1 epidemic will be focused on PrEP in combination with treatment. The PrEP drugs used in the trials were Gilead's Viread (tenofovir) and its combination drug Truvada (emtricitabine and tenofovir disoproxil fumarate), which the firm supplied for both studies (Roehr 2011).

Given the lack of an effective vaccine on the market, emergence of drug resistance to and viral escape from virtually all known antiretrovirals as well as the raging pandemic caused by this virus, new strategies to target and block the virus are of paramount importance.

Chapter 2: The Role of Glycosaminoglycans (GAGs) in HIV-1 attachment

Amongst the molecules that bind to HIV-1 are proteoglycans (PG), a cell surface component, used by many pathogens (viruses, bacteria, parasites) for attachment to the host cell. PGs are complex glycoproteins which are ubiquitous in mammalian tissues; they are composed of a protein core to which one or more glycosaminoglycan (GAG) chains is/are covalently attached. These complex glycoproteins are found in abundance in the extracellular matrix (ECM) as well as on the cell surface (glycocalyx) where they play essential roles in multiple biological processes due to their strategic placement at the interface of the communication between the cell and the external signaling environment. There is no blue print or code for the composition of GAGs, yet they are necessary for a myriad of essential processes (migration, adhesion, proliferation differentiation, coagulation, hydration, embryo development, tumor growth and pathogen attachment and entry) (Bernfield, Gotte et al. 1999; Perrimon and Bernfield 2000; Spillmann 2001; Sasisekharan, Shriver et al. 2002; Whitelock and Iozzo 2005; Sarrazin, Lamanna et al. 2011). In order to be implicated in such a vast array of processes, it is no surprise that GAGs bind to a plethora of different proteins (growth factors, cytokines, morphogens, enzymes, structural proteins, viral envelopes or capsid proteins etc) and these interactions seem to be coordinated and regulated. This is why the complexity of GAGs is so intriguing and needs to be better understood.

2.1 The Glycosaminoglycan Families

2.1.1 Galactosaminoglycans and Glucosaminoglycans

Proteoglycans can be classified according to the nature of the polysaccharide chains that are covalently attached to the protein core; PG attached to chains of heparan sulphate are referred to as (HSPG), attached to chondroitin sulphate (CSPG), attached to dermatan sulphate (DSPG) or keratan sulphate (KSPG). Serglycine is a single PG to which chains of heparin are attached, which is found in connective tissue as a specific highly sulphated HS and found in mucosal tissue as CS. As for the core proteins, they determine the localisation and the degree of expression of the polysaccharide chains. The PG's are generally classified into four large families based on their spatial placement; the membrane PGs, the extraceluular matrix PGs, the intracellular PGs and the circulating PGs and their molecular masses vary between 32 – 500 kDa (Esko and Selleck 2002). Heparan sulphates found in the intracellular space are attached to serglycine, HS found at the membrane is generally associated with syndecans, glypicans, betaglycans and CD44 isoforms. HS can also be associated with perlecan, agrine and collagen XVIII found in the extracellular matrix.

On the other hand, GAGs are long polysaccharides characterised by a repeating non-hydrolysable core disaccharide motif comprising one hexuronic acid (either a βD glucuronic acid [GlcA] or an αL iduronic acid [IdoA]) and a hexosamine

(either a glucosamine [GlcN] or a galactosamine [GalN]). With such a basic starting unit, an enormous molecular diversity is generated on three different levels for GAGs; firstly, the length of these chains can vary (chain lengths can range from few to - 25 000 disaccharide units) as well as the structural modifications (*N*- and *O*-sulphations and epimerisations [see below]) and thirdly the number and combinations of sulphated regions along an oligosaccharide chain can vary (Figure 2.1). If we look at the disaccharides in more detail, six members of the glycosaminoglycans emerge, grouped into two main veins; the galactosaminoglycans and the glucosaminoglycans (Figure 2.1).

The galactosaminoglycans comprise chondroitin sulphate (CS) and dermatan sulphate (DS) and are polysaccharides composed of repeating *N*-acetylated galactosamine (GalNAc) units associated with a glucuronic acid (GlcA) (as is the case for CS), linked together through a β1-3 liaison. In DS, the C5 carbon of some of the glucoronic acid is epimerised into iduronic acid (IdoA). Chondroitin sulphate, as their name depicts, are the GAGs that are found in large amounts in connective tissues and cartilage. They play an important role in resisting compression in certain tissues due to their elastic properties. CS are variably sulphated along their length and thus give rise to different types of CS; the most abundant dissacharides (~82%) are the monosulphated ones; when the carbon (C) 4 of the *N*-acetyl galactosamine is sulphated this is CS type A (CSA) and when sulphated on C6, this is CS type C (CSC). The non-sulphated form constitutes 11-12% and the more rare form of CS which has been initially identified in sharks and certain crustaceans called 'di-sulphated' with both C2 and C6 sulphations (CSD), C4 and C6 sulphations (CSE) and C2 in the uronic acid and C4 of the galactosamine (CSB). It can also be found in mammals (Sugahara, Masuda et al. 1991; Sarrazin, Lyon et al. 2010).

Dermatan sulphate has been considered a sub-class of CS, thus it can also go by the name of CS type B. This ambiguity is due to the fact that DS posseses a structure much like that of CS (as it contains many glucuronic acids along the length of its chain), however, DS also posseses iduronic acid due to the C5 epimerisation. This epimerisation of the C5 from glucuronic to iduronic acid favours for subsequent *O*-sulphation on the uronic acid at C2; and in order for a favourable epimerisation reaction to occur, the C4 should be preferentially *O*-sulphated. This difference in the epimerisation of the hexuronic acid C5 may be a subtle structural difference, however, it can determine protein ligand specificity as DS can bind to heparin cofactor II and CSA cannot (Mascellani, Liverani et al. 1993).

The glucosaminoglycans comprise hyaluronic acid (HA), heparan sulphate (HS) and heparin (hexosamines linked to a hexuronic acid). HA is a GAG found in conjunctive tissues, epithelium and nervous tissues, vitreous humour, synovial fluids and the skin. Its function is to maintain the hydration of the extracellular matrix and is also implicated in physiological and pathophysiological processes such as cellular adhesion, migration, tumour growth etc…). HA is not linked to a protein core and has the most simple structure out of all the GAGs as it is not sulphated; it is composed of a glucuronic acid (GlcA) linked via a β1-3 liaison to a *N*-acetyl glucosamine (GlcNAc).

Keratan sulphate was first discovered in cornea extracts in 1939 and once it was characterised in 1953 (Meyer, Linker et al. 1953), was found to be composed of a *N*-acetyl glucosamine linked via a β1-4 liaison to a glucose (instead of a hexuronic acid). Interestingly, both of the sugars can contain *O*-sulphations at position C6. Three different types of KS have been characterised; namely KS type I which is mainly found in the cornea and cartilage, KS II and KS III. They differ by the way in which they are linked to their protein cores; KSI is linked through an *N*-sulphated asparagine, however KSII and KSIII are linked through *O*-sulphated serines or threonines respectively. KSIII can also be linked to its core protein through a mannose residue (Krusius, Finne et al. 1986).

Heparin and heparan sulphate (HS) are the GAGs that possess the highest degree of sulphation and have the highest degree of structural complexity which is why they bind to the largest array of proteins. It is for this reason that heparin and HS have vast repertoires of biological and therapeutic activities and are of such great interest. This is a main topic of the current work and hence it will be described in further detail in section 2.1.2.

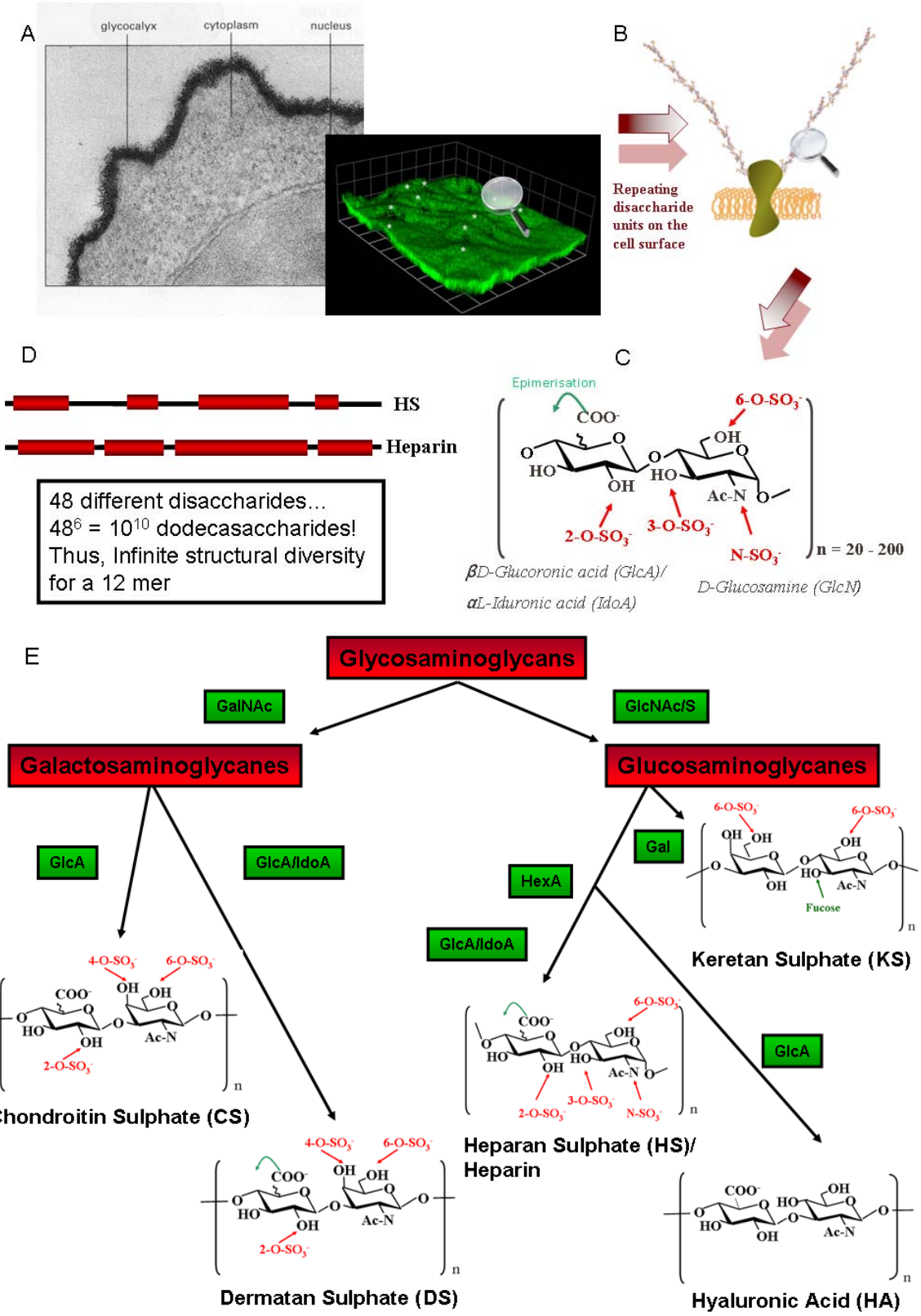


Figure 2.1 (A) This is an electron micrograph depicting a lymphocyte cell stained in ruthenium red showing the thick glycocalyx layer, which can reach up to 0.5µm. This is the interface through which the cell conducts its liaison for all biological processes (Alberts, Johnson et al. 2002). Heparan sulphates were immunostained with FITC-labelled antibodies and the image was obtained using a confocal microscope (Stevens, Hlady et al. 2007). (B) Glycosaminoglycan chains are shown covalently attached to their protein core imbedded in the cell membrane. (C) The HS disaccharide unit composed of a hexuronic acid and an N-acetylated glucosamine (4GlcA1-4GlcNAc 1) is repeated n times and can contain the

following modifications: a de-acetylation of the GlcNAc and sulphation at this residue, sulphations at positions 3 and 6 on the GlcNS and on position 2 of the hexosamine and the C5 of the uronic acid can undergo epimerisation and change from a glucuronic acid (GlcA) to an iduronic acid (IdoA). (D) Domain organisation of HS and Heparin. Highly sulphated domains (NS domains - red) are the main component of heparin, and are less frequent in HS, where there is a larger occurrence of non-sulphated domains (NA domains). The domain organisation is cell-specific and HS can be modified on so many levels, the structural diversity is vast and thus a vast number of protein binding sites exist. (E) The Glycosaminoglycan (GAG) family. GalNAc: *N*-acetyl Galactosamine, GlcNAc/S: *N*-acetyl / *N*-sulpho glucosamine, HexA: Hexuronic Acid, Gal: Galactose, GlcA: Glucuronic Acid, IdoA: Iduronic Acid.

2.1.2 Heparin and Heparan Sulphate

Heparin was discovered by accident by Mc Lean in 1916 when he demonstrated the anticoagulant activity of material fractionated from liver (hepatocytes) (Mc Lean 1916). This activity is linked to the ability of Heparin to bind and activate antithrombin III, thus inhibiting Factor Xa and thrombin. Heparin was being used to treat pulmonary emboli in the 1930's through intravenous injections and by the 1970's it was being administered by sub-cutaneous injection (McLachlin, Carroll et al. 1970).

Both heparin and HS have elevated sulphation levels and are comprised of the repeating disaccharide composed of a glucuronic acid (GlcA) linked to a N-acetylated glucosamine (GlcNAc) linked via a α1-4 liaison (4GlcA1-4GlcNAc1). Both units of the disaccharide can be enzymatically modified during their biosynthesis to contain different modifications. More precisely, these modifications include; *N*-deacetylation/*N*-sulphation of the glucosamine (GlcNAc to GlcNS), C5 epimerisation of glucuronic acid (GlcA) to iduronic acid (IdoA) and variable number of sulphations at position C2 of the GlcA(2S)/IdoA(2S) or positions 6 [GlcNAc(6S) or GlcNS(6S)] and 3 [GlcNS(3S) or GlcNS(6S,3S) (rare)] of the glucosamine (GlcN) residue. In addition, the Glucosamine can sometimes be non-substituted at the amine position, giving rise to free GlcN. So with all these modifications, 48 different disaccharide units can be generated which suggests an inconceivable structural diversity along the length of these polysaccharides leading to an equivalent functional diversity (Figure 2.1).

Sulphations occur in certain regions/domains along the polysaccharide chain. There are two types of domain; N-acetylated glucosamine (NAc domains) or N-sulphated glucosamine (NS domains) and mixed NA/NS domains have properties in between the two. In HS, about 30 - 70% of the chains consist of NS domains, however the sulphation level in heparin is significantly higher with about 80% attributed to NS domains. The major disaccharidic motif found in heparin is [IdoA 2S – GlcNS 6S].

Despite their similarities, heparin and heparan sulphate have different functions and are synthesized in different locations. Heparin is mostly expressed in mastocytes in connective and mucosal tissues and HS are expressed in all cells. Heparin is expressed in the proteoglycan form (M_r 750 000 kDa – 100 000 kDa) and many chains can be attached to serglycin, the core protein. Once the synthesis is complete, smaller fragments (M_r 5000 – 25 000 kDa) of heparin chains are cleaved at random points and stored in granules that will be secreted into the

cytoplasm of the mastocytes or outside of the cell. On the other hand, HS are rarely found as free oligosaccharide entities and are mostly attached to their core proteins (HSPGs) in the extracellular matrix and at the surface of cells (glycocalyx).

2.2 Biosynthesis and Degradation of GAGs

2.2.1 Biosynthesis and organisation

As explained above, many different members of the GAG family exist (HP/HS, CS/DS, KS and HA) and each GAG differs according to its structure, size, place of biosynthesis, post-synthesis modifications, functions and localisation. In order to produce such diversity among the different GAG families, there is a large range of specific enzymes that orchestrate the finely controlled process of GAG biosynthesis, post-synthesis modifications and proteoglycan turnover depending on the microenvironment. The extent of GAG sulphation is what largely governs their protein binding and modulating properties; thus their synthesis, structure and renewal is highly regulated in order to fine tune biological processes.

GAG biosynthesis can be divided into two groups; GAGs that are not linked to a core protein during chain elongation (HA) and GAGs that are synthesized from an anchorage point, the protein core during chain synthesis (HP/HS, KS, CS/DS).

GAG synthesis is a complex and highly regulated process and it can be broken down into three main steps; i) Initiation: formation of the tetrasaccharide linker which allows the attachment of the polysaccharide chain onto the core protein at a dipeptide serine-glycine; ii) Polymerization: then follows the synthesis of an immature saccharide chain (pro-heparan) consisting of GlcA and GlcNAc residues exclusively (for HS); iii) Polymer Modification: finally maturation of the chain occurs. For HS, N-deacetylation/N-sulphation of the glucosamines, C5 epimerisation of the GlcA to IdoA, 2-*O*-sulphation of the IdoA and 6-*O*-sulphation (and eventually 3-*O*-sulphation) of the glucosamines, takes place (Esko and Selleck 2002; Merry and Gallagher 2002; Rabenstein 2002; Kusche-Gullberg and Kjellen 2003; Lindahl and Li 2009).

2.2.1.1 Formation of the tetrasaccharide linker

Except for hyaluronic acid, biosynthesis of all GAGs is initiated by the formation of an *O*-glycosidic bond between the hydroxyl of the serine (occasionally a threonine) side chain in the core protein and a xylose in the tetrasaccharide motif GlcA(β1-3)Gal(β1-3)-Gal(β1-4)-Xyl(β1-O)-Ser (Lindahl and Hook 1978; Kjellen and Lindahl 1991; Esko and Lindahl 2001; Zhang 2010). The UDP-xylose is the donor which is transferred to the serine by xylose-transferase (XylT-1 and XylT-2) and this occurs in the endoplasmic reticulum. Following the xylose transfer, galactosyltransferases (GalT1 and 2) add the two galactoses and finally glucuronyltransferase (GlcAT-I) adds the glucuronic acid (Esko, Kimata et al. 2009) (Figure 2.2).

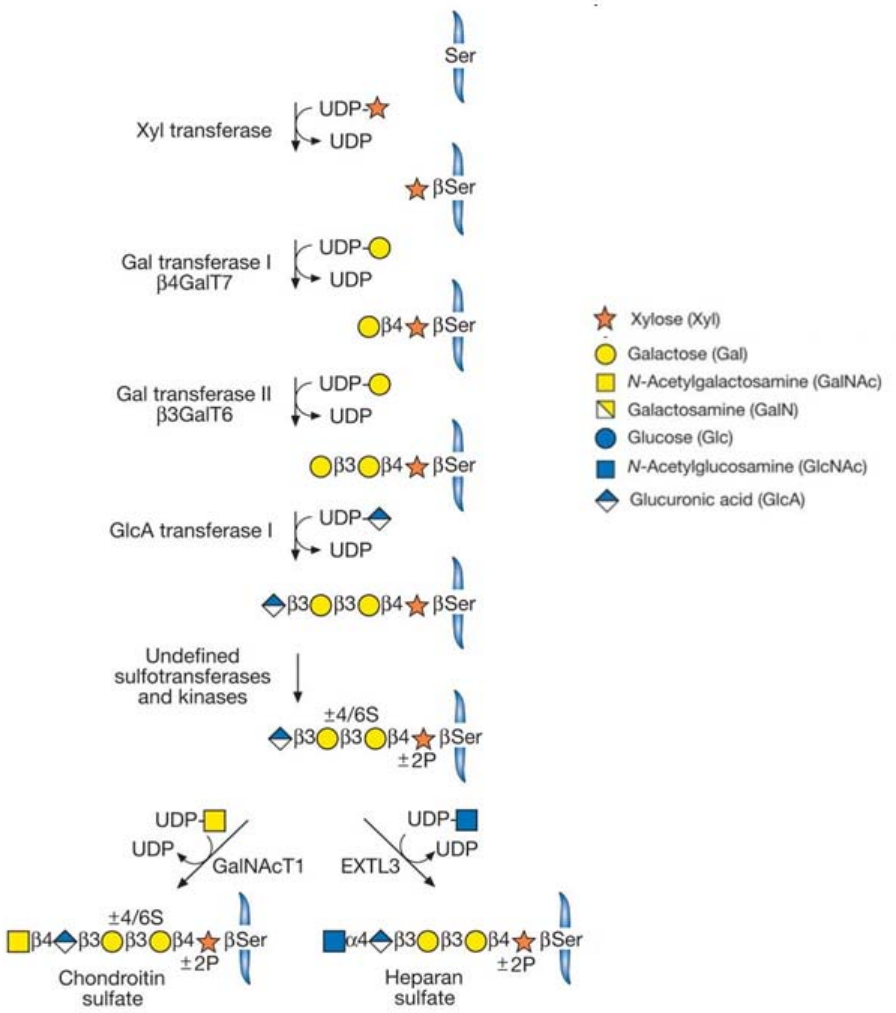


Figure 2.2 The biosynthesis of CS (*left chain*) and HS (*right chain*) is initiated by the formation of the tetrasaccharide linker between the core protein ser-gly and the polysaccharide chain. Addition of the first hexosamine decides weather the chain becomes CS (GalNAc) or HS (GlcNAc). Taken from (Esko, Kimata et al. 2009)

2.2.1.2 Chain Elongation

The next step in the biosynthesis is the subsequent addition of a hexosamine (either a galatosamine [αGalNAc] or a glucosamine [βGlcNAc]) and this will orient the biosynthesis in the direction of either CS/DS assembly or HS assembly respectively. The following saccharide that is added, determines the type of newly synthesized GAG chain that will be created. A GlcNAc will prime the synthesis of an HS chain, however a GalNAc will prime the CS/DS formation. This process and its regulation is not fully understood. For many years, the mechanism that determines the choice of synthesis of either a glucosaminoglycan or a galactosaminoglycan was unknown, however studies have shown that the structure of the core protein, the neighboring acidic residues, hydrophobic amino acids and the spacing of glycosylation sites all influence glycosaminoglycan assembly (Esko and Zhang 1996).

The discovery of GlcNAc Transferase I (GlcNAcT 1) and chondroitin GalNAc transferase (chondroitin GalNAcT 1 or 2) has also shed light on this matter. Many enzymes are implicated in this process; addition of the glucosamine is performed by enzymes EXTL 2 and EXTL 3 and addition of a galactosamine is executed by enzymes GalNAcT 1 and GalNAcT 2 (Figure 2.3) (Rohrmann, Niemann et al.

1985). EXTL 2 is a homologue of GlcNAcT I which adds a GalNAc with a α1-4 liaison onto the GlcA residue situated at the reducing end of the tetrasaccharide, however in order to initiate CS/DS synthesis a β1-4 liaison is required. Up to now, the biological role of this enzymatic activity of EXTL2 has not yet been determined.

These enzymes initiate the biosynthesis of HP/HS chains or CS/DS chains, however the choice of which type of polysaccharide chain to add does not depend on which enzymes are present at the time in the environment but rather on the amino acid sequence around the serine of the core protein to which the chains are being attached.

HP/HS polymerization begins with the alternating addition of GlcA and GlcNAc to the non-reducing end of the chain by the enzymes EXT1 and EXT2 in the golgi apparatus where they form the HS polymerase (McCormick, Duncan et al. 2000). CS/DS polymerization has taken many years to clearly understand; the chondroitin synthase (ChSy) has different enzymatic activities (β1-3-GlcA and β1-4-GalNAc transferase) and is responsible for CS chain polymerization (Kitagawa, Uyama et al. 2001).

2.2.1.3 Chain Maturation

The last stage of polysaccharide biosynthesis is an ordered process of chain modification catalysed by several different enzymes, which will generate mature and structurally diverse polysaccharides.

In HS, the first step is the prerequisite for all further modifications, it is the replacement of the acetyl group on the glucosamine for a sulphate group. These two reactions are catalysed by *N*-deacetylase / *N*-sulphotransferase (NDST) of which there are four members in humans (NDST1-4). NDST1 and NDST2 have broad expression patterns (found in most cell types and tissues), however NDST3 and NDST4 have a much more restricted expression pattern (Aikawa, Grobe et al. 2001). The first modification that takes place is extremely important for the downstream maturation of the GAG chain. Since NDST is the first-acting enzyme, it 'defines' the size and number of S domains along the GAG chain length and thus influences the action of all the sequential enzymes implicated in the GAG maturation. NDST influences the degree of modification/sulphation of the polysaccharide and can thus regulate the principal criteria that distinguish heparin from HS. In fact, NDST exerts its action at the point of divergence between HP and HS and despite the importance if its role, very little is understood about the mechanisms of its regulation. On heparin polymers, most of the GlcNAc will be N-deacetylated and then N-sulphated by the NDST. However, in the case of heparan sulphate, only a few GlcNAc residues will be modified by NDST.

Presto *et al .,* unexpectedly discovered that NDST1 competes with EXT1 for binding to EXT2 and that in the absence of EXT1, there is increased NDST1 expression, increased NDST1 glycosylation and thus a resulting increased HS sulphation (Presto, Thuveson et al. 2008). In this work, they show beautifully how depending on the different ratios of EXT1, EXT2 and NDST1 present in the

GAGosome (physical complex of enzymes committed to HS assembly), the fate of the HS chain structure will change.

The next steps of maturation are the catalysis of glucuronic acid (GlcA) to iduronic aid (IdoA) by the C5 epimerase (recently renamed Hsepi). This reaction is reversible and thus GlcA and IdoA residues are in equilibrium. The presence of IdoA favors the 2-*O*-sulphation of the uronic acids (by 2-OST) however, the addition of a 2-*O*-sulphation is an irreversible reaction and thus the IdoA 2S epimer is no longer in equilibrium with GlcA (Bernfield, Gotte et al. 1999; Li, Gong et al. 2003). The C5 epimerase also has substrate specificity as its activity depends on the presence of N-sulphated glucosamines and this is what explains the absence of IdoA in NAc domains. These two enzymes work together in concert and previous work has suggested that they exist as a heterodimer in the golgi apparatus.

Following the 2-*O*-sulphation, the HS polysaccharides are then sulphated at the C6 position of the glucosamine by a family of enzymes called 6-*O*-sulphotransferases (6-OST) of which there are 3 isoforms (6-OST1, 2 and 3). This modification is not strictly dependent on the preceding modifications and the 6S sulphation can be added onto either an *N*-acetylated glucosamine or an N-sulphated glucosamine in the transition domains. Thus far, the substrate specificity for 6-OST is not clearly known, however, it is understood that 6-OST 1 is responsible for the 6-*O*-sulphation of HS in most tissues (Habuchi, Nagai et al. 2007).

A final and rare step is the 3-*O*-sulphation of the N-sulphated glucosamines by one of 7 isoforms of 3-*O*-sulphotransferases (3-OST). This modification is important for the anticoagulant properties of heparin and HS and is required for antithrombin III fixation and it has been shown to play a crucial role in HSV entry (Yabe, Shukla et al. 2001).

All of these modifications result in the production of polysaccharides with an enormous structural heterogeneity which allows these anionic molecules to bind to an array of ligands with great specificity (Figure 2.3). The NS domains which carry a strong negative charge interact with basic regions on protein ligands and this will be discussed further in section 2.3.

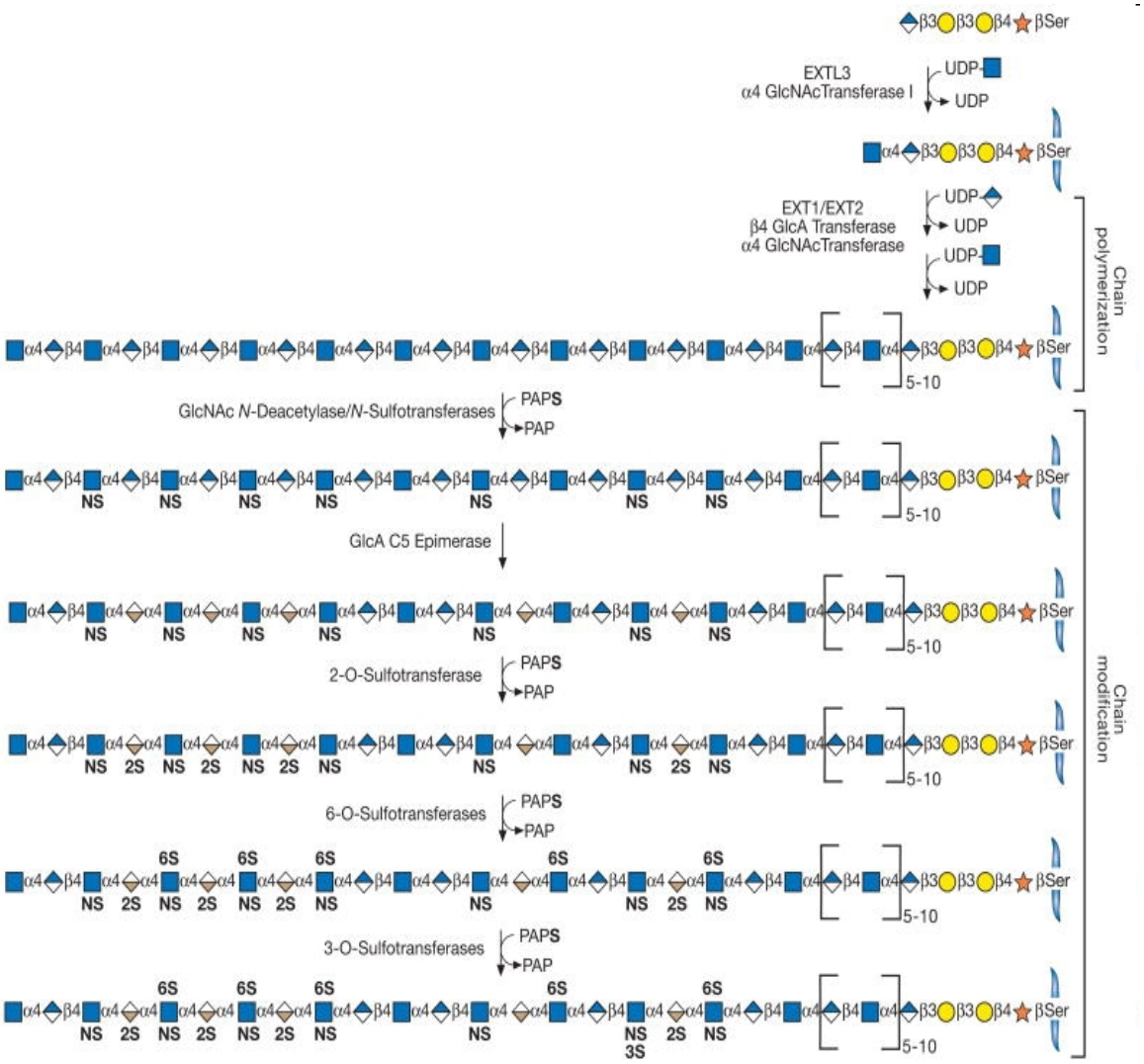


Figure 2.3 Heparan sulphate biosynthesis involves copolymerization of *N*-acetylglucosamine and glucuronic acid residues. A series of modification reactions including sulphation and epimerization of glucuronic acid to iduronic acid occurs; chain polymerization and modification are thought to occur simultaneously (PAPS) 3′-phosphoadenyl–5′-phosphosulfate, the high-energy donor of sulphate groups. Taken from (Esko, Kimata et al. 2009)

2.2.2 GAG Catabolism: Remodelling and Recycling of GAGs

In order for the cell to adapt to the rapid changes in its environment, the size, composition and structure of the proteoglycans and HS chains can be controlled by remodelling of the sulphate profile or recycling of the chains to liberate free disaccharides. It is important to distinguish between the two types of GAG catabolism; either the GAGs are functionally remodelled in order to refine their biological activity or they are physically removed (half life 3-4 hours) and then renewed at the cell surface (Yanagishita and Hascall 1984).

The classic pathway for HS degradation is the endocytosis of proteoglycans. These internalized proteoglycans are initially degraded by proteases that cleave the core protein and then hydrolases (either exoglycosidases or endoglycosidases) such as heparanase which depolymerises the HS chains at a specific number of sites, depending on sequence (Esko, Kimata et al. 2009). These smaller degraded oligosaccharides eventually appear in the lysosome and undergo complete

degradation by way of a series of exoglycosidases and sulphatases which digest the chains into monosaccharides and inorganic sulphates (Brauker and Wang 1987). Recycling of GAGs is a natural and essential process for the normal functioning of the cells. Mutations and or defects in the GAG catabolism machinery genes provoke an accumulation of partially degraded HS fragments and lead to pathologies such as mucopolysaccharidoses (MPS), rare autosomal recessive diseases that provoke irreversible lesions in the cells, tissues and organs (Ashworth, Biswas et al. 2006).

The remodelling of GAGs involves heparanases, which also have an extracellular activity, where they are capable of remodelling the HSPGs secreted to the surface of cells during inflammation, angiogenesis and metastatic tumour growth (Ihrcke, Parker et al. 1998; Dempsey, Plummer et al. 2000; Sanderson, Yang et al. 2004; Vlodavsky, Abboud-Jarrous et al. 2006). For this reason, heparanases have become a major target for anti-cancer drugs (Ferro, Hammond et al. 2004; Miao, Liu et al. 2006; McKenzie 2007). Sulfs are a family of sulphatases that have been recently discovered and they are localised on the surfaces of cells and found in the extracellular matrix after being secreted from the golgi (Dhoot, Gustafsson et al. 2001; Morimoto-Tomita, Uchimura et al. 2002). Two forms of Sulfs exist in humans (HSulf-1 and HSulf-2), and they posses an endo-glucosaminyl-6-O-sulphatase activity. Numerous studies have shown that these enzymes which modify the specific profile of the 6-*O*-sulphation within the NS domains of HS can thus significantly alter the biological properties of these HS chains and in turn alter their capacity to modulate the activity of a number of chemokines, cytokines and morphogens (Ai, Do et al. 2003; Viviano, Paine-Saunders et al. 2004; Uchimura, Morimoto-Tomita et al. 2006). Despite the great interest that these enzymes provoke in the context of cell signalling regulation and perhaps viral-host cell interaction, very little is known about their structure-function relationship and the regulation of their function.

In the case of syndecan, matrix metalloproteinases (MMP) cleave the extracellular domains from the protein at a site close to the cell membrane during periods of injury or stress (Li, Park et al. 2002) and liberate soluble syndecan into the biological milieu. Interestingly, the soluble fragments can have a completely different function from that of the syndecans immobilized in the cell membrane; for example soluble syndecan inhibits Fibroblast growth factor 2 (FGF-2), however, the cell-bound form activates it's signalling (Bernfield, Gotte et al. 1999).

2.2.3 GAG degradation enzymes in the laboratory

Enzymes that are capable of digesting GAGs have been discovered in bacteria and these have been commercialized to be used for the purpose of GAG characterization in the laboratory. These enzymes recognize precise oligosaccharide sequences and this allows the removal, identification and isolation of specific structural sub-domains of GAG chains. Heparinases I, II and III digest HP and HS between the glucosamine and the uronic acid, and chondroitinases ABC digest chondroitin sulphate A, B and C between the *N*-acetylated galactosamine and a glucuronic acid (Figure 2.4).

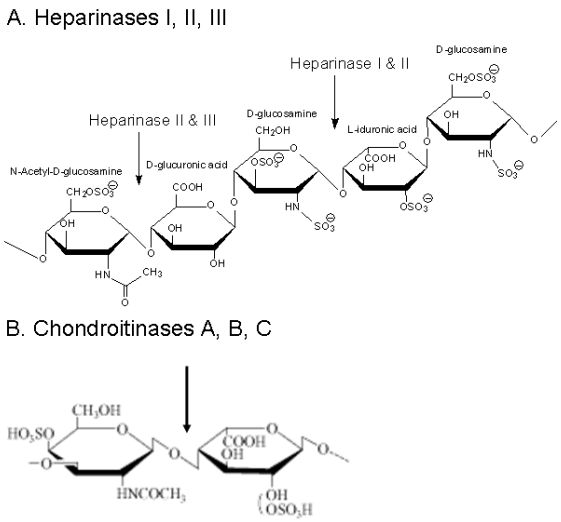


Figure 2.4 (A) The heparinases cut the oligosaccharide at the α1-4 glycosidic liaison between a glucosamine and a uronic acid (GlcA or IdoA). Heparinase I cuts between a hexosamine and a 2-*O*-sulphated uronic acid. Heparinase II cuts between a hexosamine and a uronic acid and heparinise III cuts between a hexosamine and a glucuronic acid. (B) Chondroitinase ABC cut between the N-acetyl hexosamine and the uronic acid.

2.3 HS-Protein Interactions

Since GAGs are ubiquitous (present on the surface of almost all cells and in the extracellular matrix), it is natural that these poly-anionic molecules interact with a plethora of different proteins. The study of these interactions (structural studies, dynamics and functional studies) is paramount to understanding the biological phenomena associated with GAGs as well as harnessing their properties for therapeutic applications.

2.3.1 Structure-Function Relations

The binding interaction between proteins and heparan sulphate oligosaccharides is primarily electrostatic, involving interactions between cationic side chains within the protein (ammonium, guanidinium or imidazolium groups of lysine, arginine or histidine) and anionic sites on the HP or HS. Hundreds of diverse proteins have been identified as 'heparin-binding proteins' (HBP) such as enzymes, enzyme-inhibitors, cytokines, morphogens, growth factors, matrix proteins, lipoproteins and proteins associated with disease etc (Kjellen and Lindahl 1991; Bernfield, Gotte et al. 1999; Bishop, Schuksz et al. 2007) (Table 3).

Due to their strategic placement, GAGs are implicated in an enormous amount of biological processes and communication between cells; they induce conformational changes in certain proteins (as is the case for antithrombin III) (Petitou, Casu et al. 2003) allowing for target protein recognition (a protease as is the case for antithrombin III) (Olson, Bjork et al. 1992), they participate in protein-protein interactions, they function as coreceptors (as in the case for FGF) (Rapraeger, Krufka et al. 1991), and they can also act as a site of anchorage for certain pathogens, such as gp120 on HIV-1 (Moulard, Lortat-Jacob et al. 2000). GAGs are also implicated in cell adhesion and matrix assembly (Okamoto, Bachy et al. 2003) as well as the localisation and concentration of chemokines (e.g.

CXCL12) (Amara, Lorthioir et al. 1999; Sadir, Baleux et al. 2001; Lortat-Jacob, Grosdidier et al. 2002; Sweeney, Lortat-Jacob et al. 2002), cytokines (IFNγ) (Lortat-Jacob, Kleinman et al. 1991) and the protection of certain proteins from proteolysis (Lortat-Jacob, Baltzer et al. 1996; Sadir, Imberty et al. 2004).

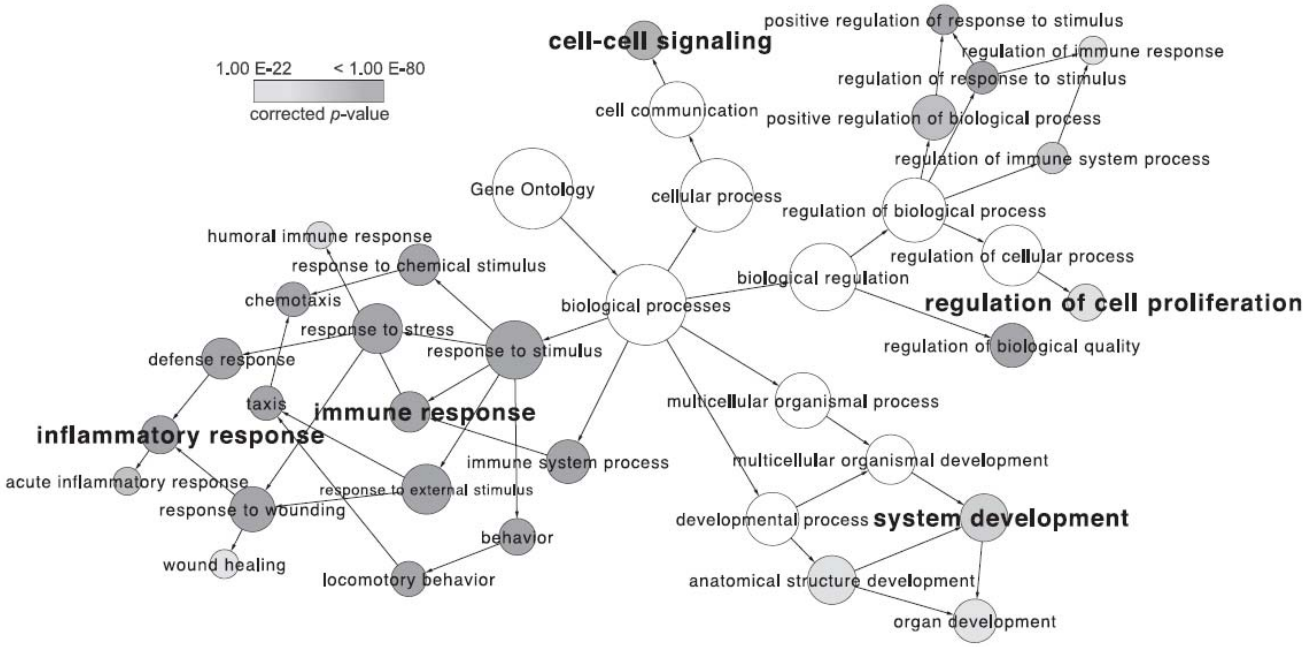


Figure 2.5 This is a gene ontology depicting the vast number of biological processes implicated in heparin/HS interactions, and thus called the 'interactome'. Data for this map was provided from different databases of interacting proteins (e.g. NCBI Entrez GeneID). The node size is proportional to the number of heparin-binding proteins belonging to the functional category and the node shade or grey indicates the statistical significance (p value) of each pathways' over representation (enrichment) in heparin binding proteins (HBP). I.e. the lighter the node, the stronger the enrichment in the interactome and the more studied the process is. Taken from (Ori, Wilkinson et al. 2011)

Table 3. Table showing the wide range of proteins that bind to heparin and heparan sulphate adapted from (Capila and Linhardt 2002; Ori, Wilkinson et al. 2011)

Family of proteins	Protein	Function
Protease/Esterase	AT-III, SLPI, C1i, VCP, trypsin-like serine proteases, subtilase family	Coagulation, metabolic pathways and the complement pathway, protein maturation by peptide bond cleavage
Growth Factors	FGFs, VEGR, HGF, PDGF, Insulin-like Growth Factor binding protein, TGF-β propeptide	Regulation of cell proliferation, differentiation and cell migration, chemotaxis, angiogenesis, cell-cell signalling
Morphogens	Wnt, Hedgehog, BMP	Development, embryogenesis, wound healing, blood vessel and vasculature development
Cytokines	IFNγ, IL-5,8,10, IL-8-like	Inflammation response, response to wound healing, defense response, immune response
Lipid-binding proteins	Annexin V, ApoE	Transport and metabolism of lipids
Adhesion Proteins	Selectins, fibronectin, Vitronectin, Collagen type V, Collagen triple helix repeat, fibrillar collagen, thrombospondin, Laminin, sushi domains	Adhesion, migration, locomotory behaviour, cell motility
Pathogens	Proteins on: HIV-1, dengue virus, HSV, papillomavirus, Adenovirus, adeno-associated virus (AAV), *streptococcus pneumoniae, plasmodium falciparum*…	Infection

2.3.1.1 Specificity

Binding interactions between heparin binding proteins (HBP) and negatively charged heparin may lead to the false perception that proteins bind HS through a poorly-specific manner. However, it has been shown that there are certain sequences within the NS and NA/NS transition domains of HS that have specific interactions with proteins and that these interactions result in a regulation of the

proteins' function (Salmivirta, Lidholt et al. 1996; Lindahl, Kusche-Gullberg et al. 1998; Turnbull, Powell et al. 2001). Such selectivity can be achieved through 'rare components' such as *N*-acetylated glucosamine (3S, 6S) present in the anti-thrombin binding pentasaccharide sequence of heparin which is essential for its anticoagulant activity (Lindahl, Kusche-Gullberg et al. 1998). Typically, between 3-7 disaccharides are involved in protein-binding, however, longer fragments can also be involved as they wrap around the protein (Lortat-Jacob, Turnbull et al. 1995; Lindahl, Kusche-Gullberg et al. 1998; Turnbull, Powell et al. 2001).

After comparing heparin binding and non-heparin binding proteins, different consensus sequences for GAG binding based on clustered basic amino acid residues were identified, including; XBBXBX, XBBBXXBX and XBBBXXBBBXXBBX, where B and X are basic and neutral/hydrophobic amino acid residues, respectively (Cardin and Weintraub 1989; Sobel, Soler et al. 1992). In addition, the HP binding sites are not necessarily linear but can also include conformational epitopes comprising distant amino acids organized in a precise spatial orientation through the folding of the protein. This has been shown by several different techniques; site directed mutagenesis (Yamashita, Beck et al. 2004), structural characterisation of protein/heparin complexes by NMR (Kuschert, Hoogewerf et al. 1998), X-ray crystallography (Mulloy and Linhardt 2001), molecular modelling (Lortat-Jacob, Grosdidier et al. 2002) and the development of a new approach, which relies on the proteolytic digestion of protein/heparin complexes and the subsequent identification of the heparin bound peptides by N-terminus sequencing, (Vives, Crublet et al. 2004).

2.3.2 GAGs as coreceptors and Internalisation

GAGs used to be thought of as low affinity receptors, and over time they have been better defined to act as coreceptors; GAGs collaborate with conventional cell-surface receptor proteins, both in binding cells to the extracellular matrix and in initiating the response of cells to some growth factors.

One of the most studied interactions between GAGs and proteins, is that of fibroblast growth factor (FGF) and heparan sulphate. Here, HS acts as a coreceptor and of the 23 different FGFs, FGF-1, 2 and 7 are the most studied. Yayon *et al.,* showed that FGF-2 requires HS in order to bind to its receptor (FGFR1) and proposed that FGF underwent a conformational change when it bound to HS, allowing recognition of its receptor (Yayon, Klagsbrun et al. 1991). In the same year, Rapraeger *et al.,* proposed that FGF forms a ternary complex with HP/HS and its receptor which consequently transmits the signal (Rapraeger, Krufka et al. 1991). Later in 1992, the controversial issue of how the FGF signalling complex is formed was further developed by Ornitz and colleagues, where they proposed that an octasaccharide HS is the minimum size to bind and cause the dimerisation of two FGFs, which then causes dimerisation on the receptors and downstream signalling (Ornitz, Yayon et al. 1992). It is now accepted after further structural studies that HS reinforces the interaction between the FGF-FGFR complex 2:2 and that this dimerisation of the receptors transduces the signal that leads to autophosphorylation of the kinase. Here, GAGs are shown to act as coreceptors and stabilize protein-protein interaction.

As mentioned above, GAGs are recycled by a means of internalisation and downstream transport into lysosomes in the cytoplasm where they are subsequently digested. During internalisation of the GAGs, proteins that are bound or associated with the GAGs will be cointernalised, such is the case for vitronectin, thrombospondin, FGF2, ATIII as well lipoprotein lipase (LPL). LPL is an enzyme produced by adipocytes and is essential to lipid metabolism. The monomeric form of LPL has a weak interaction with cell surface GAGs, however as a dimer it interacts strongly with HS with a low nM affinity (Lookene, Savonen et al. 1997). When associated with the GAGs, LPL is in close proximity to the lipoproteins and thus allows digestion. When the GAGs are internalised, so are the lipoproteins and LPL, leading to their endocytosis and catabolism.

2.3.3 Capture, Release and Protection of proteins

HSPGs can transiently capture growth factors and morphogens which may stabilize protein gradients to control the range of signalling (Lander, Nie et al. 2002; Guimond and Turnbull 2004; Koziel, Kunath et al. 2004; Kirkpatrick and Selleck 2007) or protect proteins against degradation by proteases (Small, Nurcombe et al. 1992). On the other hand, proteins that are bound to the GAGs may be released through protease-mediated shedding of the PG ectodomains or through cleavage of the HS chains by heparanase (Lindahl and Li 2009). HSPGs can also act in *trans*, where the core protein of one HSPG attached to one cell will extend and deliver a protein to a 'receptor' located on a neighbouring cell. Through a balance of these signalling and structural roles, HSPG signalling coreceptors can either be tumour promoting or tumour suppressing and understanding the mechanism of action of these processes will enable effective targeting of the coreceptors and pathways for treatment of human disease.

2.4 Role of HS in pathogenic Infections

Other than their implication in a multitude of biological processes, HS are also exploited by a large number of pathogens as a site of anchorage onto the host cell from which they can gain entry. Pathogens use HS as an attachment site to increase the concentration of infectious particles at the surface of the cell as well as to spatially facilitate easy access to the host's coreceptors.

2.4.1 Attachment of bacteria and parasites

Parasites such as *Plasmodium falciparum* (responsible for malaria), *Trypanosoma cruzi* (responsible for Chagas disease) and *Toxoplasma gondii* (causative agent for encephalitis) use GAGs to gain entry into host cells. *P.falciparum* binds to chondroitin sulphate A chains on the cells in the salivary gland in the mosquito (Barragan, Spillmann et al. 1999). GAGs are also a site of attachment for bacteria such as *Pseudomonas aeruginosa*, *Helicobacter pylori*, *Listeria monocytogenes*, *Streptococcus pneumoniae*… Some bacteria exploit the properties of oligosaccharide fragments as tools to augment their virulence. As in the case for *P. aeruginosa,* invasion of the host cell provokes signalling pathways that involves tyrosine kinases activation and downstream release of syndecans into the extracellular milieu (Schmidtchen, Frick et al. 2001). These liberated

polysaccharides are thus participating in the virulence of *P. aeruginosa* and are capable of binding to and inactivating host cationic anti-microbicidal peptides such as defensins. Conversely, mutant knock-out mice for the expression of syndecan-1 are more resistant to infection by *P. aeruginosa* than their wild type counterparts (Park, Pier et al. 2000).

2.4.2 Attachment of viruses

Many viruses are capable of binding to GAGs; HIV, herpes (HSV-1), adenovirus, papillomavirus and dengue virus. Primarily, HS serves as an attachment receptor that enables these viruses to concentrate on the host cell surface and gain proximity to the actual cell coreceptors and may influence viral tropism *in vitro* (Ugolini, Mondor et al. 1999; Spillmann 2001; Germi, Crance et al. 2002; Liu and Thorp 2002). Enzymatic treatment of T-cell lines with heparinases and removal of sulphates from GAG chains with sodium chlorate prevents HIV-1 (IIIB/Hx10) infection (Patel, Yanagishita et al. 1993; Mondor, Ugolini et al. 1998). Here, I will focus on the interaction between HS and HIV throughout the course of infection and the therapeutic applications of HS.

2.4.2.1 HS binding to gp120

HIV is able to bind to a range of molecules present on the cell surface, other than its classical primary receptor and coreceptors, CD4 and CCR5/CXCR4 respectively (Ugolini, Mondor et al. 1999). As described in Section 1.2.10, such molecules are lectins, DC-SIGN, glycosphyngolipides and GAGs, particularly heparan sulphate.

Like other pathogens (Vives, Lortat-Jacob et al. 2006), HIV exploits the properties of HS to attach to the cell surface however, the exact role of these polysaccharides during HIV infection remains unclear. Because of their abundance, HS were mainly considered as "attachment receptors" of the virus, the function of which was to facilitate the infection by concentrating viral particles on the surface of the host cell. This can be referred to as infection in *cis*. Various studies have shown that the elimination of HS expressed on the cell surface enabled these cells to become less permissive to infection, at least with lab-adapted viruses (Roderiquez, Oravecz et al. 1995). In parallel, HS can mediate infection in *trans*, whereby HS expressed on non-permissive cells can bind to and collect the virus at the cell surface and then transfer the virus to permissive cells (Olinger, Saifuddin et al. 2000; Bobardt, Saphire et al. 2003).

HIV-HS interactions can occur at different times in different situations and serve different purposes. At the point of HIV entry, the virus encounters host mucosal surfaces and the abundant HS molecules in the glycocalyx trap the viral molecules and efficiently allow the translocation of the virus through the epithelial layer towards their target cells (Bomsel and Alfsen 2003; Wu, Chen et al. 2003). In a similar fashion, HS expressed by endothelial cells of the blood-brain barrier sequester the viral particles and contribute to the process of neuro-invasion (Argyris, Acheampong et al. 2003; Banks, Robinson et al. 2004). Due to their anionic nature, HS will preferentially bind to CXCR4 utilizing HIV envelopes due to their overall basic charge and thus HS may play an important role in viral tropism during the course of infection.

The interaction between HIV-1 and HS depends on the quality and degree of sulphation of the oligosaccharides present at the cell surface and these parameters depend on the tissue of origin and the cell's state of activation and differentiation (Maccarana, Sakura et al. 1996; Ohshiro, Murakami et al. 1996). Most of the research around HIV-1 and HS binding is conducted using cells that express high levels of HS, however, the natural hosts of HIV-1 do not express such high levels. $CD4^+$ T Lymphocytes express weak amounts of HS which may vary as a function of their stage of differentiation and monocytes also express low amounts of HS. However, macrophages express low levels of CD4 and high levels of HS and it has been suggested that HS can compensate for low levels of CD4 on macrophages (Saphire, Bobardt et al. 2001). A study by Bobardt *et al.*, characterising the oligosaccharide motifs that are involved in HIV-1 fixation onto HS has shown that the 6-*O*-sulphate residues are crucial for viral binding to epithelial cells (Bobardt, Chatterji et al. 2007). The characterisation of this binding interaction is highly complex and is vital to better understanding viral entry and the development of entry inhibitory compounds.

2.4.2.2 Characterisation of the gp120/HS interaction

In 1995, Roderiquez *et al.,* showed that HIV-1 gp120 bound to heparan sulphate through its V3 loop (Roderiquez, Oravecz et al. 1995), however the structural and functional aspects of this interaction were not well understood. Moulard and colleagues showed that this interaction was largely due to the electrostatic attraction between the anionic HS on the cell surface and the global basic charge of the V3 loop (Moulard, Lortat-Jacob et al. 2000). A R5 HIV-1 variant typically possesses a net V3 loop positive charge of ≤ 5 and that of an X4 variant is typically ≥ 5 (Briggs, Tuttle et al. 2000). The prediction of CCR5 coreceptor usage from HIV-1 *env* sequences, is performed by the presence of neutral and negatively charged amino acids at positions 11 and 25 respectively, in the V3 loop (De Jong, De Ronde et al. 1992; Fouchier, Groenink et al. 1992; Milich, Margolin et al. 1993; Fouchier, Brouwer et al. 1995; Xiao, Owen et al. 1998; Hoffman, Seillier-Moiseiwitsch et al. 2002). Conversely, if a basic/positive amino acid is found at position 11 and/or 25 the sequence will probably represent a SI phenotype (De Jong, De Ronde et al. 1992; Fouchier, Groenink et al. 1992; Fouchier, Brouwer et al. 1995; Milich, Margolin et al. 1997; Brumme, Dong et al. 2004; Brumme, Goodrich et al. 2005). Such small variations in the charge of the V3 loop can have great consequences for the affinity of the envelope for HS.

2.4.2.2.1 Gp120 binding to HS is linked to Tropism

CCR5 utilizing envelopes do not bind to HS as strongly as do CXCR4 utilizing envelopes, however, how this phenomenon is linked to early-stage infection is not fully understood. This has been confirmed in our laboratory when injecting different tropic gp120 over a surface of immobilized heparan sulphate using Surface Plasmon Resonance (SPR, section 8.10.5) technology. X4-tropic envelopes have a high affinity for the immobilized HS, shown by the low dissociation of the stable complex that is formed (Figure 2.6) and R5-tropic gp120 binds with a much lower affinity than the X4-tropic envelopes due to the reduced number of positive charges in its V3 loop. As can been seen here, there is correlation between viral tropism and the ability for gp120 to bind to HS and since

there are such great differences between the two types of virus (their pathogenicity, distribution during infection), HS is likely to play a major role in these differences. Interestingly, the same effect is seen for whole viruses; X4 viruses also form a stable complex when injected over an HS surface and R5 viruses bind very weakly and dissociate off an HS surface (Moulard, Lortat-Jacob et al. 2000).

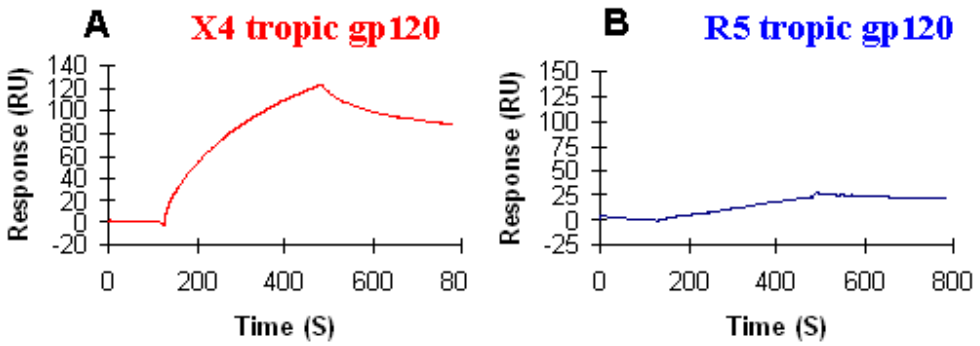


Figure 2.6 Surface Plasmon Resonance (SPR) binding curves showing the interaction between X4 (A) and R5 (B) tropic envelopes binding to a HS surface. 60nM of each envelope was injected and negative binding surface data was subtracted. Taken from (Lortat-Jacob, Fender et al. 2005). The binding responses (in RU) were recorded as a function of time (in s) – these parameters will be used for all SPR sensograms throughout the manuscript.

2.4.2.2.2 The CD4 induced (CD4i) domain is an HS binding site

As mentioned above, the V3 loop was established as being a point of attachment between the HS and gp120. In 2005 Vivès *et al.,* showed that the CD4i site was a second binding site on gp120 for HS when they injected gp120 (HxBC2) either in the presence or absence of CD4 on an immobilized HS surface using an SPR-based technique (Biacore) (Vives, Imberty et al. 2005). The gp120/CD4 complex had a much higher binding response for the HS as compared to the gp120 on its own (Figure 2.7 A and B). This result was confirmed by injecting gp120/CD4 complexes over a 17b surface in the presence and absence of heparin. The monoclonal antibody 17b is used as a coreceptor surrogate as it belongs to a group of monoclonal antibodies defined as induced by CD4 (Thali, Moore et al. 1993), the epitopes of which on gp120 overlap the coreceptor binding surface.

The gp120/CD4 complex binding to 17b is clearly inhibited by the presence of heparin, thus heparin binds to the coreceptor binding region of gp120 and inhibits recognition by the 17b antibody (Figure 2.7 C).

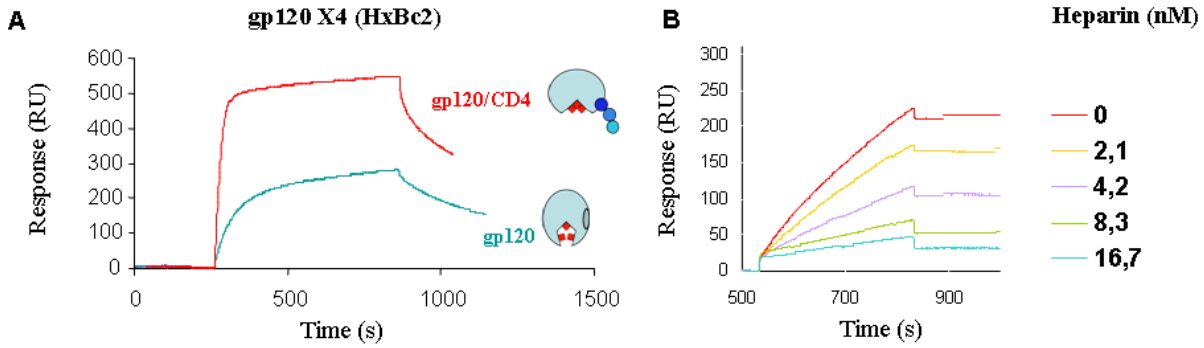


Figure 2.7 (A) 50nM of either gp120 alone (blue curve) or gp120 in the presence of equimolar amount of CD4 (red curve) over a Heparin surface. (B) Inhibition of gp120/CD4 (5 and 10nM respectively) complex binding to 17b on the sensor chip surface in the presence of different concentrations of heparin (0-16.7nM).

Molecular modelling has confirmed that an oligosaccharide can indeed interact with both the V3 loop and CD4i site simultaneously. The model suggests that the size of the oligosaccharide that is required to cover all the basic residues of these two domains is between 10-12 monosaccharides long (Figure 2.8A). This model was confirmed by inhibiting the binding of gp120/CD4 complexes onto a 17b surface with varying sizes of purified heparin oligosaccharides (dp [degree of polymerisation] 2 – 18). The experimental data was in clear agreement with the modelling data as an oligosaccharide of at least 10 monosaccharides is necessary to significantly inhibit the gp120/CD4 complex from binding to its coreceptor surrogate 17b (Figure 2.8B) (Vives, Imberty et al. 2005).

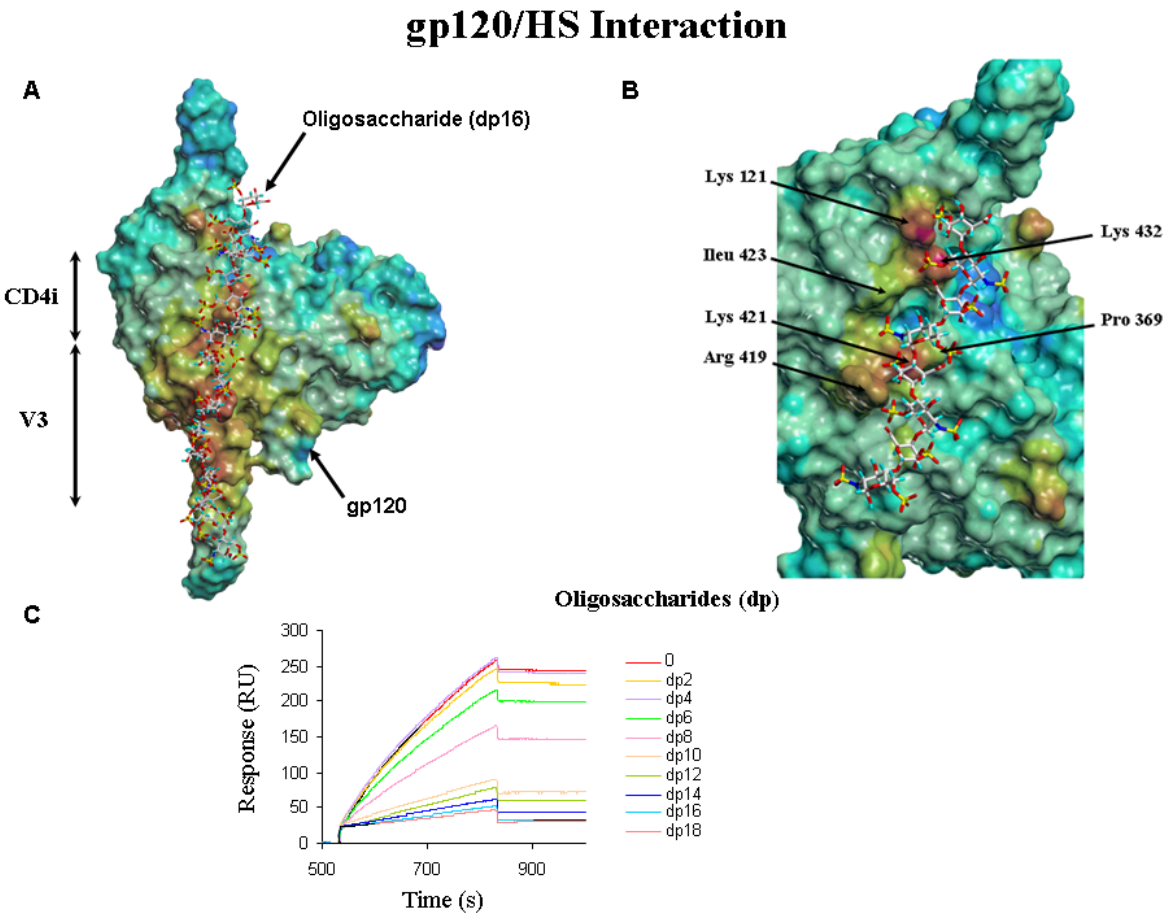


Figure 2.8 (A) Molecular modelling of the placement of a hexadecasaccharide of heparin onto the gp120 (HxBC2) crystallographic structure, showing that a dodeccasaccharide encompases both the V3 and CD4i binding sites. The MOLCAD surface of the gp120 is coloured according to its electrostatic potential (red for the basic residues and blue for the acidic residues). (B) Zoom up of the CD4i site with the basic amino acids involved in the GAG- binding interaction annotated. (C) The gp120/CD4 complexes (5 and 10nM respectively) were co-incubated with different lengths of heparin oligosaccharides before injecting them over a 17b surface on the Biacore. A decasaccharide is the smallest fragment required for significant inhibition of the complex binding to 17b. Images adapted from (Vives, Imberty et al. 2005).

In order to investigate the structural basis of the HS-gp120 interaction in more detail, Crublet *et al.*, used a mapping strategy and compared the heparin binding activity of wild type and mutant gp120 molecules using SPR based binding assays (Crublet, Andrieu et al. 2008). Through the use of several gp120 constructs containing mutated amino acids in the CD4i region, combined with the mapping strategy, four heparin binding domains were identified: In the V2 and V3 loops, in the C-terminal domain and within the CD4-induced bridging sheet. Three of these regions are areas that undergo structural re-arrangements upon the binding of CD4 and are involved in co-receptor recognition. Residues Arg419, Lys421 and Lys432 are all involved in coreceptor recognition and are targeted by heparin and these sites are targets for viral entry inhibition (Rizzuto, Wyatt et al. 1998).

2.5 Therapeutic applications of HS in HIV infection

2.5.1 Anionic Binders

One of the promising strategies to combat HIV-1 entry is with the use of sulphated polysaccharides as they have been shown to inhibit HIV-1 infection (Ueno and Kuno 1987; Baba, Snoeck et al. 1988; Bagasra and Lischner 1988). Polyanions recognise proteins via the clusters of basic amino acids exposed on their surfaces (Whitelock and Iozzo 2005) and thus a large body of work has characterised the gp120/HS complex, showing that heparin, HS, polyanions and dextran sulphate bind to the V3 loop of gp120 and can compete with V3 loop specific monoclonal antibodies (Callahan, Phelan et al. 1991; Batinic and Robey 1992; Rider, Coombe et al. 1994; Okada, Patterson et al. 1995).

The bridging sheet and epitopes exposed upon CD4 binding (CD4i) are also involved in the binding of polyanions (Roderiquez, Oravecz et al. 1995; Moulard, Lortat-Jacob et al. 2000). The CD4i can also bind to negatively charged sulphotyrosine residues found in the complementary determining region of CD4i neutralizing monoclonal antibodies (412d and E51) and also found in the N-terminal of both coreceptors, CCR5 and CXCR4 (Choe, Li et al. 2003; Huang, Venturi et al. 2004).

Many sulphated polysaccharides have thus been studied for their antiviral properties and potential usage as microbicides. Sulphated polysaccharides extracted from marine sponges (*Erylus discophorus*) show strong anti viral activity (up to 95% inhibition of HIV-1) (Esteves, Nicolai et al. 2011). Dextran sulphate can possess 2-3 sulphate groups per D-glucose α (1-6) and can bind to V3 loop and CD4i of gp120 in the same fashion as HS (Callahan, Phelan et al. 1991; Moulard, Lortat-Jacob et al. 2000). It is important to note that CCR5 utilizing gp120 do not fix as strongly to these polyanions as do CXCR4 utilizing gp120 or dual tropic ones (Moulard, Lortat-Jacob et al. 2000). The bacterial derived lipopolysaccharide (LPS) has been shown to bind the V3 loop and inhibit gp120 binding to coreceptors and inhibit infection of U87 cells by pseudoviruses. Such a compound could potentially be used as an anti-HIV-1 therapy (Majerle, Pristovsek et al. 2011).

As mentioned above, the CCR5 coreceptor of HIV-1 posses a certain number of sulphated tyrosines (in particular at positions 10 and 14) (Farzan, Mirzabekov et al. 1999; Cormier, Persuh et al. 2000) in its N-Terminal. (Section 1.2.5 – HIV coreceptors). Interestingly, several human HIV-1 neutralizing antibodies that are directed against the CD4i site in gp120, contain sulphotyrosines in their heavy chain CDR3 regions (complementary determining region 3) of their antigen binding sites (including mAb 412d, E51) which are crucial for binding to gp120 and neutralizing infection (Choe, Li et al. 2003; Huang, Venturi et al. 2004; Huang, Lam et al. 2007). Recently, broadly neutralizing antibodies PG9 and PG16 have been described to also contain sulphotyrosines (Walker, Phogat et al. 2009; Pejchal, Walker et al. 2010).

Dorfman *et al.,* described a tyrosine sulphated peptide derived from the heavy chain CDR3 region of E51 and showed that it bound and neutralised HIV-1 more

efficiently than sulphotyrosine-containing peptides directly based on the CCR5 amino terminus sequence (Dorfman, Moore et al. 2006). This may be due to the peptides' increased flexibility and solubility. Due to the conserved gp120 sulphate-binding domains, this peptide is able to bind and neutralise both R5X4 and X4 isolates.

Very recently, Kwong *et al.*, showed that when a 15 amino acid peptide mimetic of the CCR5 coreceptor was fused to a CD4 mimetic peptide and a dimeric antibody Fc domain (DM1-Ig), it can bind gp120 and neutralise R5, X4 and R5X4 HIV-1 isolates (Kwong, Dorfman et al. 2011). Despite DM1-Ig promising results, it neutralises HIV-1 with much less efficiency than certain neutralizing antibodies and it does not neutralize many non-clade B isolates; thus improvements are necessary for this compound to be used *in vivo*. Here, the binding assays between gp120 proteins and the inhibitors were performed with immunoprecipitation experiments which do not yield accurate results compared to more sensitive techniques such as Surface Plasmon Resonance. In addition, the infection assays were not performed against whole infectious virus, but against co-transfections of gp120-encoding plasmids and NL4-3-encoding plasmids lacking *env* and *nef* and expressing GFP (Kwong, Dorfman et al. 2011).

In conclusion, the concept of cooperatively inhibiting HIV-1 entry with a chimeric inhibitor capable of binding both i) the CD4 binding domain as well as ii) the CD4i region is novel, and most importantly it *does* inhibit both R5 and X4 viral entry into host cells. The challenge is to design and construct a molecule, with a high affinity and capacity to block binding to these two critical regions, that is not degraded upon administration, has little to no side effects and is economically viable.

2.5.2 Concept and action of CD4-HS: a glycoconjugate that inhibits HIV-1 attachment and entry

From the above data, the role played by HS interacting with the CD4i during HIV-1 infection is not known. However, this conserved and cryptic domain (CD4i) is involved in the recognition of CCR5 and CXCR4, and observations described above strongly suggest a strategy to inhibit the interaction between gp120 and the coreceptor, based on the use of an HS oligosaccharide. In order for the cryptic coreceptor binding domain CD4i to be exposed and neutralised, gp120 first needs to bind CD4. Based on these facts, a unique strategy was formed to target HIV-1 entry: once gp120 has bound CD4, the conformational re-arrangement occurs in the gp120 protein which exposes/creates the cryptic CD4i which is favourably positioned to bind the coreceptor (CCR5/CXCR4) immediately after CD4 binding. Thus the concept of creating a bivalent inhibitory molecule (CD4-HS) that will trigger the gp120 conformational change and block viral entry. The CD4-HS molecule is able to initially bind the CD4-binding site on gp120 (with a CD4 moiety), thereby exposing the CD4i domain, after which the second part of the inhibitory molecule (the HS anionic moiety) is perfectly positioned to tightly bind the CD4i, thus blocking the gp120 from binding the host coreceptors.

Thus, with the idea of replacing full length CD4 with a small synthetic peptide mimetic, a chimeric molecule was conceptualised and constructed: mCD4-HS_{12} (Baleux, Loureiro-Morais et al. 2009). This chimeric molecule is composed of a 27 amino acid long peptide, mimicking CD4 (mCD4), covalently linked to a HS_{12} and operates in the following way (Figure 2.9).

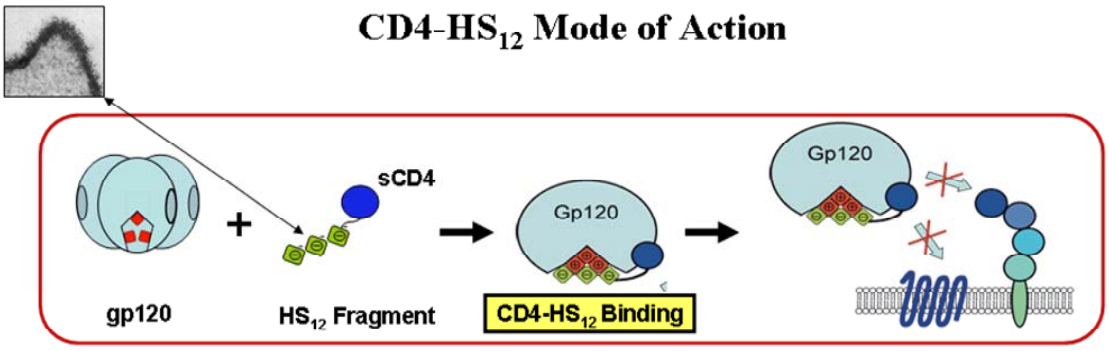


Figure 2.9 CD4-HS_{12} mode of action: the glycoconjugate binds gp120 through the CD4 moiety which then induces the formation of the coreceptor binding domain via the synthetic CD4, followed by the high affinity interaction of the anionic HS_{12} domain with coreceptor binding domain. The glycoconjugate blocks both CCR5 and CXCR4 viral entry.

2.5.2.1 The CD4 moiety, mCD4

Instead of using the full length CD4 receptor, a mimetic approach was used whereby CD4 was replaced by a peptide that was based on a short mini CD4, called CD4M33 (Martin, Stricher et al. 2003). In 2003, Martin *et al.,* published the design of CD4M33, a 27 amino acid CD4 mimetic that contains the minimal amount of residues to obtain the optimal interaction with gp120 in order to unmask conserved neutralization epitopes on gp120 that are normally cryptic on the unbound protein (Martin, Stricher et al. 2003). After the structure resolution of CD4-gp120-17b antibody complex (Kwong, Wyatt et al. 1998), the ~800Å CD4 binding pocket in gp120 was elucidated, revealing the Phe43 hydrophobic pocket. This structural insight spurred the design of CD4M33 which has potential to be used in vaccine formulations in complex with envelope proteins or for the use as a molecular target in phage display technology to develop broad-spectrum neutralizing antibodies.

2.5.2.2 The HS Moiety, HS_{12}

A heparan sulphate dodecasaccharide (obtained by chemical synthesis) was created as this length of oligosaccharide was optimal for CD4i binding based on the SPR data as well as molecular docking experiments. Each repeating disaccharide contains three sulphates, namely the *N*-sulphate and the 6S sulphate on the Glucosamine and the 2S sulphate on the Iduronic acid. Thus, in total there are 18 sulphate residues found in the HS_{12} synthetic prototype (Baleux, Loureiro-Morais et al. 2009).

2.5.2.3 mCD4-HS_{12}

Since the CD4 induced coreceptor binding site that is exposed upon CD4 binding is in such close proximity to the host membrane, neutralising antibodies are sterically inhibited from accessing the conserved and vulnerable region (Labrijn, Poignard et al. 2003). Hence, the ingenious idea of linking the miniCD4 molecule to the relatively small HS moiety. Owing to the covalent bond between the mCD4 and the HS_{12}, the glycoconjugate mCD4-HS_{12} has a very high affinity for the viral envelope. It simultaneously blocks the interaction of gp120 MN (CXCR4-tropic) and YU2 (CCR5-tropic) with three of its ligands: HS, CD4 and CD4i antibodies (17b, 48d and X51) (Baleux, Loureiro-Morais et al. 2009). The antibodies were used as coreceptor mimics and thus validated the mode of action described above; however the CD4-HS_{12} molecule, at this stage had never been tested using actual 7 transmembrane GPCR coreceptors as gp120 ligands.

This molecule is a strong HIV-1 entry inhibitor candidate as it inhibits attachment and entry of both CCR5 tropic and CXCR4 tropic HIV-1 strains with 1-5nM IC_{50} for inhibition of viral replication (Baleux, Loureiro-Morais et al. 2009). Other advantages of the glycoconjugate is that it interacts with the virus and not the host cells and due to its unique mode of action, it simultaneously inhibits two important regions of gp120 crucial for entry; the CD4 binding region and the coreceptor binding domain. Drug resistance to this molecule is unlikely to occur as the CD4 binding region is required to remain conserved in order to continue binding CD4 and the basic region within gp120 required for the HS interaction is also conserved and crucial for the interaction with the coreceptors. Any mutations that might occur in these two regions are likely to be detrimental to the survival of the virus, thus this inhibitor has enormous potential not to evoke resistance. The concept of cooperatively inhibiting viral entry through the simultaneous blockage of two different critical domains required for viral entry, opens a novel strategy for viral entry inhibition in general.

However, the limitations of mCD4-HS_{12} are its inherent complexity and extreme difficulty in synthesis of the anionic dodecasaccharide moiety. A novel strategy to mimic the anionic moiety was thus necessitated in order to reduce the complexity of the molecule for structure-function analysis and also to reduce the time required for the molecules' synthesis/production. Therefore, in order to screen for various HS mimetics, a screening platform was required whereby HIV-1 envelope binding to its native coreceptors could be monitored and inhibited by various inhibitors. Native coreceptors are advantageous over soluble 17b, as 17b is only a partial coreceptor mimic and in order to inhibit binding to all the coreceptor epitopes involved in 'gp120-CD4-coreceptor binding interaction', the use of full length native coreceptors is advantageous. This work involved the setting up of a coreceptor binding assay whereby native coreceptors were immobilized on a platform in a lipid-detergent buffer. In order to test whether these coreceptors were functional, the system was validated by the use of binding a natural chemokine ligand to the coreceptors. This introduces the following chapter on the CXCL12 chemokine.

Chapter 3: CXCL12 / Stromal Derived Factor 1 (SDF1), natural ligand of CXCR4

3.1 General

Chemokines are small (8-10 kDa) chemotactic cytokines which comprise a large family of about 50 members. They have been identified to bind to a total of ~20 receptors, meaning that there are many receptors which bind more than one chemokine (Figure 3.1). Chemokines and their receptors are important in dendritic cell maturation (Sozzani, Allavena et al. 1998) and T and B cell development (Forster, Emrich et al. 1994; Vicari, Figueroa et al. 1997). Thus they are essential to many developmental and physiological possesses. They direct the orientated migration of cells during development, inflammation, hematopoietic stem cell mobilization, homeostatic immune responses, organogenesis and neuronal communication. The major role of chemokines is to act as a chemoattractant to guide the migration of cells and they are the only members of the cytokine family that act on GPCRs.

Some chemokines participate in immune surveillance and are referred to as homeostatic chemokines; they are constitutively expressed and direct lymphocytes to the lymph nodes so they can search for invading pathogens by interacting with antigen-presenting cells residing in these tissues. Other chemokines have roles in development; they promote angiogenesis, or guide cells to tissues that provide specific signals critical for cellular maturation. Another group is the inflammatory chemokines released from a wide variety of cells in response to bacterial infection, viral infection or agents (eg. silica) that cause physical damage (Alberts, Johnson et al. 2002). Thus if the cell's normal ability to traffic cells is damaged or hijacked by any bacteria/virus or agent, the chemokine network can maintain and coordinate many disease states such as autoimmune diseases (multiple sclerosis, rheumatoid arthritis, and atherosclerosis) as well as in abnormal cell-growth conditions such as tumour metastasis and proinflammatory immune responses due to inappropriate cell recruitment (Baggiolini 2001; Proudfoot 2002; D'Ambrosio, Panina-Bordignon et al. 2003; Sun, Cheng et al. 2010). Many studies have shown the link between chemokines and their receptors to disease and this is all summarized in Figure 3.1. Most of the disease associations are derived from animal studies, however, some human disease samples have confirmed the animal data. Thus, chemokine signalling has been the target of drug discovery efforts almost since the initial identification of chemokines, 25 years ago, due to their negative signalling effects in disease states. These efforts have tried to identify small molecule therapeutics that target their receptors.

From the Figure 3.1, it is clearly depicted that some chemokines have a promiscuous nature where they bind more than one coreceptor and the inverse is also true where certain receptors bind more than one ligand. For this reason, the structure-function relationships between the receptor and ligands are critically important for fine-tuned, healthy signal regulation. Thus it is evident that such a delicately balanced system can have many negative repercussions.

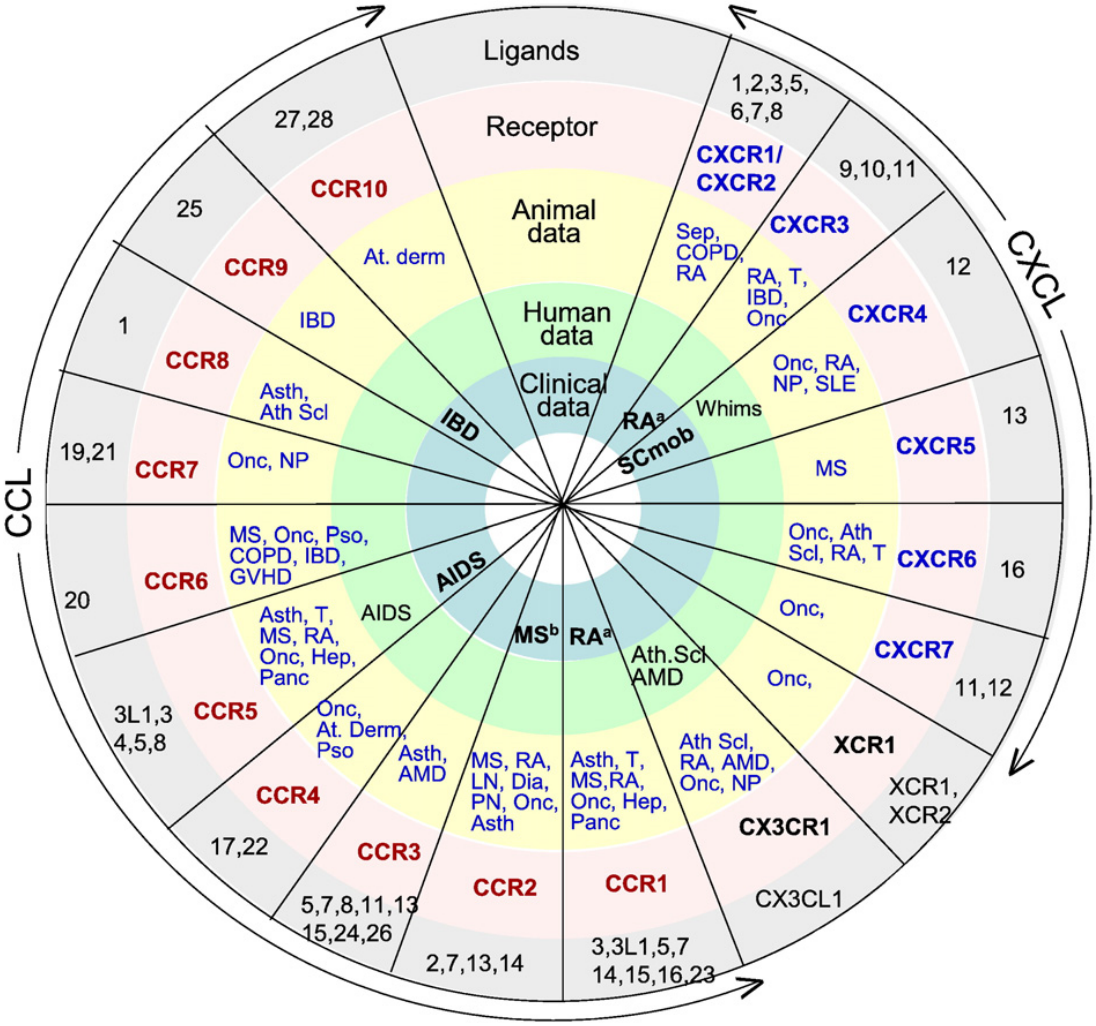


Figure 3.1The association of chemokines (outer ring in grey) and their receptors (second ring from the outside in pink) and the associated disease (first three rings from the inside towards the outside in blue, green and yellow, for clinical data, human data and animal data respectively). A selection of disease associations obtained from animal models using gene deletions, neutralizing antibodies and receptor antagonists, as well as expression data in human samples and positive results from clinical trials. abbreviations: Sep, Sepsis; RA, Rheumatoid arthritis; T, Transplant; IBD, Inflammatory Bowel Disease; Onc, Oncology; SLE, Systemic Lupus; MS, Multiple Sclerosis; Ath Scl, Atherosclerosis; COPD: Chronic Obstructive Pulmonary Disease; AMD, Acute macular degeneration; NP, Neuropathic pain; Asth, Asthma; At. Derm, Atopic dermatitis; Hep, Hepatitis; Panc, Pancreatitis; Pso, Psoriasis; GVHD, Graft vs Host disease. (Garin and Proudfoot 2011)

3.2 Chemokines - Nomenclature and classification

The nomenclature of chemokines depends on the presence and structure of a conserved first two cysteine residues in the amino-terminal region of the molecule, thus forming four families (CC, CXC, CX3C and C) (Murphy, Baggiolini et al. 2000). The position of the cysteines is denoted by whether they are adjacent, CC (as in RANTES and monocyte chemoattractant protein 1 (MCP-1) and macrophage inflammatory protein 1 (MIP-1α and β)), or are separated by

residues, CXC (as in Interleukin-8 (IL-8)), CX_3C (neuroactin/fraktalkine) or just singular, C (lymphotactin) (Baggiolini, Dewald et al. 1997; Rollins 1997). This motif is followed by an L (ligand) and finally an identifying number. Similarly, the receptors (R) for these molecules are named by the chemokine class that they recognise and are numbered in order of their discovery.

3.3 Chemotaxis

3.3.1 The Chemokine side

In terms of their structure, chemokines generally share a similar tertiary structure, despite the fact that their sequence homology is highly variable (ranging from less than 20% to over 90%). Owing to NMR and X-Ray crystallographic techniques, structures have revealed that the chemokines tertiary structure consists of a disordered N-terminus (which functions as the key signalling domain), followed by a N-loop which ends in a 3_{10} helix, followed by a three-stranded β-sheet and a small C-terminal helix (Clore, Appella et al. 1990; Lodi, Garrett et al. 1994; Skelton, Aspiras et al. 1995; Handel and Domaille 1996; Crump, Rajarathnam et al. 1998; Liwang, Wang et al. 1999; Mizoue, Bazan et al. 1999; Blaszczyk, Coillie et al. 2000; Swaminathan, Holloway et al. 2003).

After translation, chemokines are secreted from the cell in response to different stimuli where they interact with their receptors, except for chemokines CX_3CL1 and CXCL16 which are tethered to the extracellular surface. The secreted chemokines action is executed by binding to and activating specific GPCRs to induce cell migration along a gradient of increasing concentration of chemokine towards the origin or source of secretion (chemotaxis) (Rot 1993; Veldkamp, Seibert et al. 2008) (Figure 3.2). Secreted chemokines are protected from proteolysis (Sadir, Imberty et al. 2004) and prevented from diffusing away from their sites of production and dispersing under the influence of flow and retained and presented to their coreceptors by glycosaminoglycans (Ali, Palmer et al. 2000). The binding between glycosaminoglycans and chemokines (in particular CXCL12α) will be described in more detail later.

3.3.2 The Cell side

Once the chemokine has bound to its receptor, the chemokines activate cascades of complex signal transduction pathways; involving the heterotrimeric G-proteins, adenyl cyclase, phospholipases (PL), protein tyrosine and serine/threonine kinases, lipid kinases, the Rho family of small GTPases and triggering of intracellular second messengers (cAMP, phophoinositides and calcium) (Reif and Cantrell 1998; Ward, Bacon et al. 1998).

Heterotrimeric G proteins (α, β and γ subunits) are bound by many GPCRs via their C-terminal and the cytoplasmic loops. The G-protein's α subunit is bound to a GDP in its resting state, and when the GPCR is activated, the GDP is replaced by a GTP and the α-GTP complex then dissociates from the bound βγ subunits to further affect intracellular signalling proteins or target functional proteins directly depending on the α subunit type. To increase the complexity of this system,

GPCRs can associate into dimers and oligomers which enhances sensitivity and the specificity of each response (more on this later). One of the signalling pathways is the mitogen-activated protein kinase pathway (MAPK pathway) the activation of which results in the stimulation of transcription factors and the regulation of the expression of cell cycle proteins. One of the three MAPK classes is the extracellular signal-regulated kinases (ERK), the phosphorylation of which we detect in the laboratory as a sign of GPCR activation and successful cell signalling.

All these complex signalling cascades cause leukocytes to migrate from the bloodstream across the wall of microvessels to the underlying tissue which is the essential step in inflammation and response to infection. In order for a cell to initiate migration, it must undergo a polarization in its morphology which will enable it to convert cytoskeletal forces into a net cell-body displacement. These morphological changes involve the rearrangement of the cytoskeleton, changes in filamentous F-actin and the formation of integrin-mediated focal adhesions. The cell binds and detaches from the substrate in a co-ordinated manner with extension and retraction of pseudopods executing the directional migration (Bokoch 1995; Ward, Bacon et al. 1998).

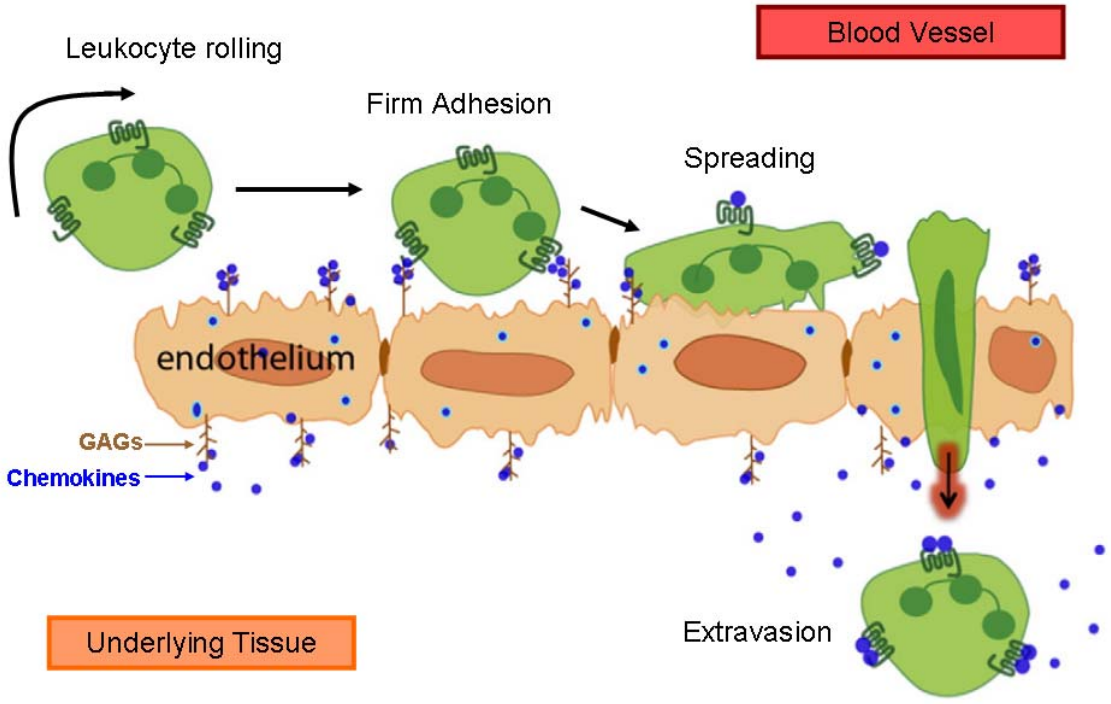


Figure 3.2 A classical cartoon depicting the basic steps in cell migration in response to chemokine production. Chemokines are presented on the endothelial surface GAGs to chemokine receptors on leucocytes in the blood; chemokines may oligomerise on the GAGs. Whether the chemokines bind simultaneously to GAGs and chemokine receptors is not yet fully understood. Leukocyte recruitment is a multi-step process involving cytokines and chemokines driving selectin-mediated adhesion, subsequent arrest, firm adhesion, rolling and transmigration. This image was adapted from (Salanga and Handel 2011).

This chapter focuses on the chemokine called stromal derived factor 1α (SDF-1α), also known as CXCL12α which is constitutively expressed in the bone marrow,

lungs and liver. This chemokine shares the same two ligands as CXCR4 utilizing gp120, which are CXCR4 and heparan sulphate. Studying the interactions between CXCL12, CXCR4 and HS can provide insight and bring about a better understanding of these two ligands which are very important for HIV-1 attachment too.

3.4 The CXCL12 chemokine

3.4.1 Gene expression of CXCL12

CXCL12α was originally identified in bone marrow stromal cells and was characterised as a pre-B-cell stimulatory factor (Nagasawa, Hirota et al. 1996). It is constitutively expressed and secreted in the bone marrow, lung, liver and lymph nodes (Zlotnik 2006) and highly conserved among mammalian species. CXCL12α orchestrates a large array of essential functions, both during embryonic development and postnatal life. This is confirmed by mutant mice (*sdf1*$^{-/-}$ or *Cxcr4*$^{-/-}$) which die *in utero* due to grave developmental defects (Nagasawa, Hirota et al. 1996; Tachibana, Hirota et al. 1998; Zou, Kottmann et al. 1998).

CXCL12 is regulated at the splicing level and not at the transcriptional level; a single mRNA strand is alternatively spliced to produce six different isoforms (α, β, γ, δ, ε, and φ) of this chemokine which have been found in humans (Yu, Cecil et al. 2006). All isoforms share the first three exons found in the α isoform (residues 1-68), however the other isoforms vary in their fourth exon at the C-terminal– thus giving rise to specialized C-terminal domains (Figure 3.3) The alternate splicing has been thought of as a natural mechanism to generate functional diversity, without structural modifications and complications (Romero, Zaidi et al. 2006). For the purpose of this study, I will only focus on CXCL12α and CXCL12γ.

3.4.2 Structure of CXCL12α

The three-dimensional structure of CXCL12α was determined by NMR (Crump, Gong et al. 1997) and by X-ray crystallography (Dealwis, Fernandez et al. 1998). CXCL12α is a monomer with a disordered N-Terminal region (Lys 1 – Tyr 8), followed by a long flexible loop, a 3_{10} helix, a triple stranded antiparalled β-sheet ($\beta_{(1)}$, $\beta_{(2)}$, $\beta_{(3)}$), covered by a C-terminal α helix $\alpha_{(C)}$. The N-terminus is anchored to the rest of the molecule by disulphide bridges (Allen, Crown et al. 2007) (Figure 3.3). Figure 3.3 and Table 4 summarise the basic sequence information of the different chemokine isoforms and mutant used in this study. The CXCL12γ isoform has an elongated 30 amino acid C-terminal with multiple HS binding domains (BBXB) witch are unique to this isoform. However, the structure-function relationships of this C-terminal are not fully defined and it is for this purpose that the biochemical studies were persued for this isoform.

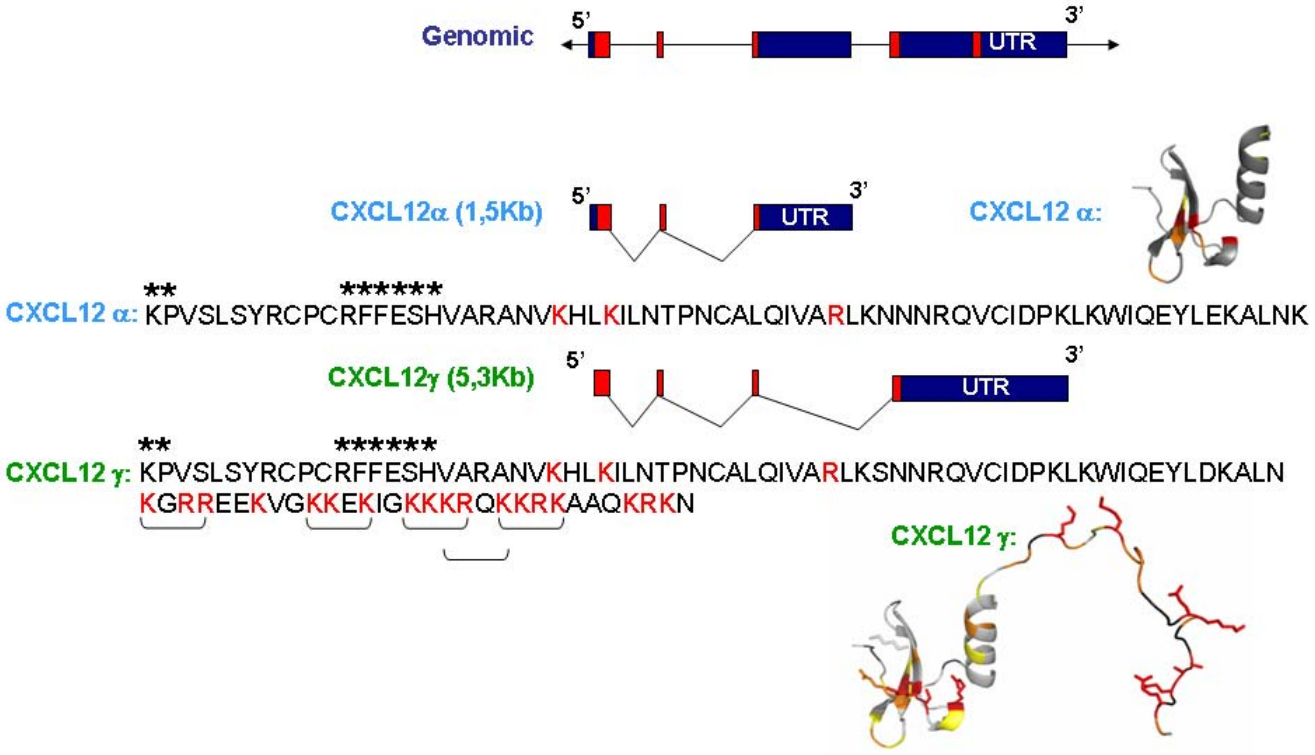


Figure 3.3 Alternate splicing of the two main isoforms relevant for this work, CXCL12α and CXCL12γ. The basic amino acids (K and R) that are highlighted in red in the sequences are amino acids that have been shown to be implicated in GAG-binding. ** KP signalling residues, **** RFFESH initial contact/docking site with receptor and GAG-binding domains (BBXB) are indicated in the CXCL12γ sequence by the black brackets and the structures of each isoform are shown. Chemical shift variations upon GAG addition (dp4) are represented on CXCL12α and CXCL12γ in colour; Red residues bind the most to GAGs and orange residues bind less and yellow residues bind the least (Laguri, Sadir et al. 2007; Laguri, Arenzana-Seisdedos et al. 2008).**

Table 4. List of physical attributes of the different CXCL12 isoforms used in this study.

	CXCL12α	CXCL12γ
# of amino acids	68	98
Molecular Weight	7835.2	11565.7
Theoretical PI	9.81	10.61

3.4.3 Physiological roles of CXCL12 and pathogenic effects

In embryonic life, CXCL12α plays non-redundant roles in the development of the cardiovascular system, the central nervous system, haematopoiesis and colonisation of the gonads with primordial germ cells (Ma, Jones et al. 1998; Nagasawa, Tachibana et al. 1998; Zou, Kottmann et al. 1998; Klein, Rubin et al. 2001; Ara, Nakamura et al. 2003). In post-natal life, CXCL12α is involved in trans-endothelial migration of leukocytes, homing and egress of stem cells from the bone marrow and their migration into peripheral tissues (Aiuti, Webb et al. 1997; Campbell, Hedrick et al. 1998). The CXCL12α / CXCR4 axis also been shown to play critical roles in pathophysiological processes such as chronic

inflammatory disorders, angiogenesis, wound healing and tumour cell proliferation (Nanki, Hayashida et al. 2000; Burger and Kipps 2006).

CXCR4 is found in cells from over 20 types of cancer which metastasize towards tissues that secrete CXCL12α (bone marrow, lung, liver, lymph nodes) (Zlotnik 2006). The current paradigm suggests that increased CXCR4 expression leads to the ability of carcinoma cells to metastasize to organs such as the bone marrow and liver that express high amounts of CXCL12α (Balkwill 2004).

However, hypermethylation of CXCL12α promoter cytosines causes the absence of CXCL12α expression which has been detected in 40-62% of tumours. This varying percentage indicates an alternate mechanism for modifying the expression of CXCL12α. A study by Wendt *et al.,* showed that an elevated migratory signalling response to ectopic CXCL12α, contributes to the metastatic potential of CXCR4-expressing mammary carcinoma cells subsequent to epigenetic silencing of the autocrine *Cxcl12* promoter (by methylation). Congruently, the re-establishment of CXCL12α production in CXCR4-expressing mammary carcinoma cells, increased proliferation and primary tumour growth and decreased chemotaxis and metastasis (Wendt, Johanesen et al. 2006; Wendt, Cooper et al. 2008). This suggests that the primary tumour cells that silence CXCL12α are at a selective advantage for metastasis through ectopic CXCL12α, and when endogenous CXCL12α is produced, proliferation is favoured and not metastasis as the cancer cell producing CXCL12α will disrupt the haptotactic gradient. This is why metastatic tumours are commonly located in organs of high CXCL12α concentration (e.g. lungs, bones, adrenal glands) and not in the organs where low CXCL12α levels are detected (heart, kidneys) (Phillips, Burdick et al. 2003).

Stem cell mobilization and hematopoietic stem cell homing to bone marrow following transplantation, are two crucial processes which are also both mediated by CXCL12α. In order for stem cells to mobilize, CXCL12α binding to CXCR4 in the bone marrow is disrupted, causing a reversion in the bone marrow-blood CXCL12α gradient (i.e. higher concentration of CXCL12α in the blood), thus resulting in the release of CXCR4 expressing cells into the blood. The cytokine called granulocyte-colony stimulating factor (G-CSF) augments the activity of certain proteases in the bone marrow which degrade both CXCL12α and, to a lesser extent, CXCR4. This is what decreases the CXCL12α binding to CXCR4 in the bone marrow, which allows for stem cell mobilization with reduced chemotactic ability due to the partial destruction of CXCR4 (Weidt, Niggemann et al. 2007). This is why the CXCR4 bicyclam antagonist AMD3100, in combination with G-CSF is used in stem-cell transplantation in patients with multiple myeloma and non-Hodgkin lymphoma who had received prior chemotherapy (Flomenberg, Devine et al. 2005). AMD3100 specifically and reversibly blocks CXCL12α from binding to CXCR4 and recently Dar *et al.,* have speculated that CXCL12α release from bone marrow cells into the circulation is caused by AMD3100 (Dar, Schajnovitz et al. 2011).

Most studies have focused on CXCL12α and its role played in normal immune functioning and disease states, however, very little is known about the CXCL12γ isoform. CXCL12α is detected in all organs, mostly those which are associated to the immune system (bone marrow, thymus, tonsils etc.), however CXCL12γ has

shown distinct mRNA expression profiles during organogenesis in several cell types during development (Franco, Rueda et al. 2009).

Few studies have demonstrated comparisons between CXCL12α and CXCL12γ signalling effects in *vivo*. Intraperitoneal administration of CXCL12α and CXCL12γ produces virtually the same local inflammatory response after 3 hours, but after 16 hours, an inflammatory reaction was still present in animals injected with CXCL12γ and not with CXCL12α (Yu, Cecil et al. 2006; Rueda, Balabanian et al. 2008). This demonstrates that the signalling of CXCL12α is much more short lived compared to the prolonged signalling effects of CXCL12γ. Structural differences between the two chemokines could explain the differences in observed functions and their relative ability to bind GAGs. This will be elaborated on later.

Shortly after CXCR4 was discovered as being a coreceptor for HIV-1, CXCL12α was subsequently reported as being the only natural ligand of CXCR4 which is able to prevent entry of CXCR4 utilizing HIV-1 through coreceptor occupancy, downregulation and internalisation (Bleul, Farzan et al. 1996; D'Souza and Harden 1996; Feng, Broder et al. 1996; Oberlin, Amara et al. 1996; Amara, Gall et al. 1997). Interestingly, CXCL12γ was shown to be the strongest HIV-1 inhibiting CXCL12 isoform (Altenburg, Broxmeyer et al. 2007).

As mentioned above, aberrant expression of chemokines plays essential roles in chronic inflammatory processes. Thus, small molecule antagonists of CXCR4 will not only inhibit CXCR4 utilizing HIV-1, but also chronic inflammatory disorders too. The functional role for having so many different CXCL12 splice variants is not fully understood and deciphering this will elucidate fundamental information on the regulation of normal and disrupted immune functioning.

3.4.4 GAGs, CXCR4, CXCR7 and CXCL12α

GAGs, CXCR4 and CXCR7 are all binding partners for CXCL12α. Once CXCL12α has been secreted into the extracellular space, it binds to the cell-surface glycosaminoglycans which play a key role in ensuring the correct positioning of the chemokine within tissues and maintaining haptotactic concentration gradients along which CXCR4 expressing cells can migrate directionally (chemotaxis). The glycosaminoglycans not only provide a scaffold for haptotactic gradient formation by chemokines, but also modify their conformation, stability, reactivity and protect the chemokines from proteolysis as well as present them to their receptors. Unlike other chemokines, CXCL12α is less promiscuous and has been know to bind only to CXCR4 and CXCR7. Thus, HIV-1's glycoprotein, gp120, and CXCL12α share a common binding partner and it is for this reason that we are interested in the CXCL12α-CXCR4 binding interaction – as it may help us gain insight into how gp120 binds CXCR4 and how this interaction may be blocked.

Recently, CXCR7 (formerly known as RDC1) has also been identified as a CXCL12α second receptor (Balabanian, Lagane et al. 2005; Burns, Summers et al. 2006; Altenburg, Broxmeyer et al. 2007). CXCR7 has phylogenetic similarity to GPCRs but does not couple G-proteins and does not induce typical chemokine receptor mediated cellular responses, however, it was found to be implicated in cardiovascular system development in CXCR7 deficient mice. In humans,

CXCR7 is expressed in embryonic neuronal and heart tissue, in hematopoietic cells and activated endothelium (Sierro, Biben et al. 2007). Elevated levels of CXCR7 expression correlate with aggressiveness of prostate cancer and promote growth and metastasis of mouse tumour models (Miao, Luker et al. 2007; Wang, Shiozawa et al. 2008). Interestingly, some studies suggest that CXCL12α signalling can be modulated through the heterodimerisation of CXCR4 and CXCR7; this will be discussed later (Sierro, Biben et al. 2007; Levoye, Balabanian et al. 2009). Also, it is important to note that the affinity of CXCL12α for CXCR7 (K_D ~ 0.3nM) is much higher than for CXCR4 (K_D ~ 4nM) (Crump, Gong et al. 1997; Balabanian, Lagane et al. 2005; Burns, Summers et al. 2006) and it has been postulated that posterior CXCR7 removal of CXCL12α sharpens chemotactic gradients promoting CXCR4-mediated migration of primordial germ cells (Boldajipour, Doitsidou et al. 2011).

Very recently, CXCR7 has been proposed as a scavenger for CXCL12α mediating ligand internalisation and subsequent targeting of the ligand for degradation (Naumann, Cameroni et al. 2010). This role may be important for the fine tuning of the mobility of hematopoietic cells in the bone marrow and lymphoid organs. The presence of 10μM AMD3100 had no effect on CXCR7-mediated CXCL12α scavenging and the deletion of the C-terminus of CXCR7, completely abolished CXCL12α degradation. This means that CXCL12α binds CXCR7 in a different way compared to how it binds CXCR4 and that the C-terminal of CXCR7 is required for trafficking of the receptor (Naumann, Cameroni et al. 2010). To date, there have not been any investigations into the role of the different CXCL12 splice variants on CXCR7 function, however, Reuda and colleagues have shown that when analysing the ability of a wild type chemokine to compete with a C-ter biotinylated CXCL12α chemokine for binding to CXCR7, both CXCL12α and CXCL12γ bind similarly to CXCR7 (IC_{50} = 6.56 nM and 10.37 nM respectively) (Rueda, Balabanian et al. 2008).

Since the role of CXCR7 in CXCL12 signalling is relatively recent and controversial, I shall only go into more detail on the interactions that have been well-described and accepted for many years. Thus, described below are the GAG-CXCL12α interactions and CXCR4-CXCL12α interactions respectively in more detail.

3.4.5 Characterisation of the GAG-CXCL12 complex

At the site of secretion, chemokines (usually highly basic proteins) are released into the extracellular space and they bind to glycosaminoglycans (high density of negative change) so as to be retained at the inflamed site creating high local concentrations of chemokine. They form concentration gradients to provide directional signals for migrating cells (Lau, Allen et al. 2004; Handel, Johnson et al. 2005; Johnson, Proudfoot et al. 2005; Proudfoot 2006). This ionic interaction between GAGs and chemokines has been demonstrated *in vitro* (Witt and Lander 1994; Hoogewerf, Kuschert et al. 1997; Kuschert, Coulin et al. 1999) and *in vivo* (Rot 1993). Cells expressing GPCRs that are specific for a certain chemokine will migrate towards the origin of that chemokine secretion. GAGs display a medium to high affinity for chemokines, and it has been hypothesized that if it were not for such immobilization, chemokine gradients would be disrupted by diffusion,

especially in the presence of flow in blood vessels and draining lymph nodes, and thus become diluted to such low concentrations that directional signals for migration cease (Johnson, Proudfoot et al. 2005). GAG-binding deficient mutants have been observed to be non-functional *in vivo* (while active *in vitro*) thus showing the importance of GAG-binding in chemokine function (Rueda, Balabanian et al. 2008; O'Boyle, Mellor et al. 2009). Chemokines which are bound to cell-surface GAGs, are then presented to their receptors, GPCRs, and induce conformational changes that trigger intracellular signalling pathways implicated in cell movement and activation, explained in more detail below.

Paradoxically, chemokines are simple and small proteins yet they orchestrate a myriad of biological functions. Thus, their interactions with GAGs explain the ability of such simple proteins to have access to such a wide range of functions. Current models suggested that GAGs enhance chemokine immobilization, local concentration, compartmentalisation, oligomerisation (Johnson, Proudfoot et al. 2005; Veldkamp, Peterson et al. 2005), formation of haptotactic gradients of the protein along cell surfaces or within the ECM, directional cues for migrating cells (Campanella, Grimm et al. 2006), protect chemokines from enzymatic degradation (Sadir, Imberty et al. 2004), and promote local high concentrations at the cell surface, facilitating receptor binding and downstream signalling. However, structurally how the chemokines react with GAGs is not fully understood.

As mentioned above, determining the binding site between a GAG fragment and its protein binding partner is complex. A technique to determine the GAG binding sites is to mutate basic residues within linear sequences which contain the typical 'GAG-binding motif', BBXB. However, despite chemokines acting as monomers under biological conditions, they tend to dimerise/oligomerise when interacting with GAGs which suggests that there may be larger or more binding epitopes involved in the context of higher order complexes when compared to a monomer (McCornack, Boren et al. 2004; Jin, Shen et al. 2007). Oligomerisation may increase the affinity for GAG binding as a larger binding surface is created which could be important in cell-surface presentation during blood flow (Salanga and Handel 2011). Another powerful way of generating diversity and specificity in chemokine-GAG interactions is through the oligomerisation of chemokines and the fact that different oligomeric states may bind to different GAG sequences (Handel, Johnson et al. 2005).

3.4.5.1 Activities of the GAG-CXCL12 complex

While immobilized heparan sulphate is essential for the biological activity of chemokines, demonstrated by inactive GAG-binding deficient mutants *in vivo* (Proudfoot, Handel et al. 2003), soluble heparin has been shown to inhibit the biological effects of chemokines as demonstrated *in vitro* (Kuschert, Coulin et al. 1999) and *in vivo* (Johnson, Kosco-Vilbois et al. 2004). In 2007, Murphy *et al.,* also showed that soluble heparin and heparan sulphate negatively affected chemotaxis *in vitro* mediated by CXCL12α and using NMR and X-ray crystallographic techniques, they show that there are two heparin binding sites on the CXCL12α dimer interacting with a heparin disaccharide; one lies at the β-strands of the dimer interface (forming hydrogen bond contacts with the following

residues: His25, Lys27 and Arg41) and the other lies at the amino-terminal loop and the α-helix (making contacts with Ala20, Arg21, Asn30 and Lys64) (Murphy, Cho et al. 2007). Taken together with the fact that treatment of cells with heparitinases (enzymes that degrade GAGs) induces a significant reduction of CXCL12α binding to cells (Mbemba, Benjouad et al. 1999; Mbemba, Gluckman et al. 2000), a mechanism can be proposed in which GAGs bind to CXCL12α dimers so as to sequester the chemokines and present them to their CXCR4 receptors, and that this mechanism is disrupted/regulated in the presence of soluble GAGs. Thus, interfering with chemokine-GAG interactions can be an effective strategy to target inflammation.

3.4.5.2 The GAG component of the complex

Heparan sulphate interacts with a large array of proteins (as discussed in Chapter 2) and it has been thought that protein recognition by HS resides within specific epitopes, are characterised by precise *N* and *O*-sulphation distributions. In order to correlate the structure of HS with their binding activities, one would require high resolution information on GAG:Chemokine interactions with oligosaccharide chains that are longer than a disaccharide, which would be more biologically relevant. For this, homogeneous compositions of GAG chains would be required for structural analysis and due to the inability to synthesize GAG from a template, challenges in synthesizing GAGs and the extreme complexity and diversity of these molecules, there is a lack of detailed molecular information on these interactions.

Chemokines display 4 classes of non-overlapping HS binding sites and thus represent a specific binding signature for each group of chemokine (Lortat-Jacob, Grosdidier et al. 2002). Of the four classes, cluster 2, which has only been observed in CXCL12α, forms a crevasse at the interface between the β-strands, where three basic amino acids in both $\beta_{(1)}$ and $\beta_{(2)}$ strands characterize the binding site. In 1999 and 2001, Amara *et al.,* and Sadir *et al.,* identified through site-directed mutagenesis and surface plasmon resonance experiments, that Lys24 and Lys27 on CXCL12α are essential for the interaction with heparin (Amara, Lorthioir et al. 1999; Sadir, Baleux et al. 2001). Arg41 and Lys43 are also involved in the interaction, however are not essential. They also showed that a minimum size of 12-14 monosaccharides are required for the efficient binding interaction and a docking study confirmed the involvement of Lys24, Lys27, Arg41 and including Lys1 in the binding for the polysaccharide (Sadir, Baleux et al. 2001) (Figure 3.4).

More recently, Laguri *et al.,* used ^{15}N-CXCL12α to titrate a solution of ^{13}C labelled octasaccharide, which is homogeneously *N*- and 6-*O*-sulphated on its glucosamine residues and unmodified on its glucuronic residues and called $dp_{8NS,6S.}$ The binding interaction was followed by multidimensional NMR spectroscopy and a structural model of the CXCL12α-HS complex was made (Laguri, Sapay et al. 2010). With this tool, a more accurate and detailed map of the GAG binding residues on chemokines can be identified. Significant chemical shifts were detected in the same HS binding site that was observed in the previous model from Sadir *et al.,* in addition to another 20 other residues on CXCL12α that occur outside the HS binding site. This observation was attributed to a heparin-

induced dimerisation event as has been observed previously (Veldkamp, Peterson et al. 2005). An advantage of performing this titration with a ^{13}C labelled octasaccharide was that the sugars which participate in the interaction were able to be defined. All the *N*-sulphated and *6-O*-sulphated glucosamine residues collectively contributed to the interaction (Figure 3.4).

Understanding how HS binds to proteins and regulates their functions is thus of great interest, however, it has been hindered by the extreme complexity and chemical heterogeneity of these polysaccharides. Using chemokines is a useful tool to study the interactions of these complex polysaccharides with proteins. An isoform of CXCL12 that is particularly interesting is CXCL12γ due to its unusual structure and high affinity for GAGs.

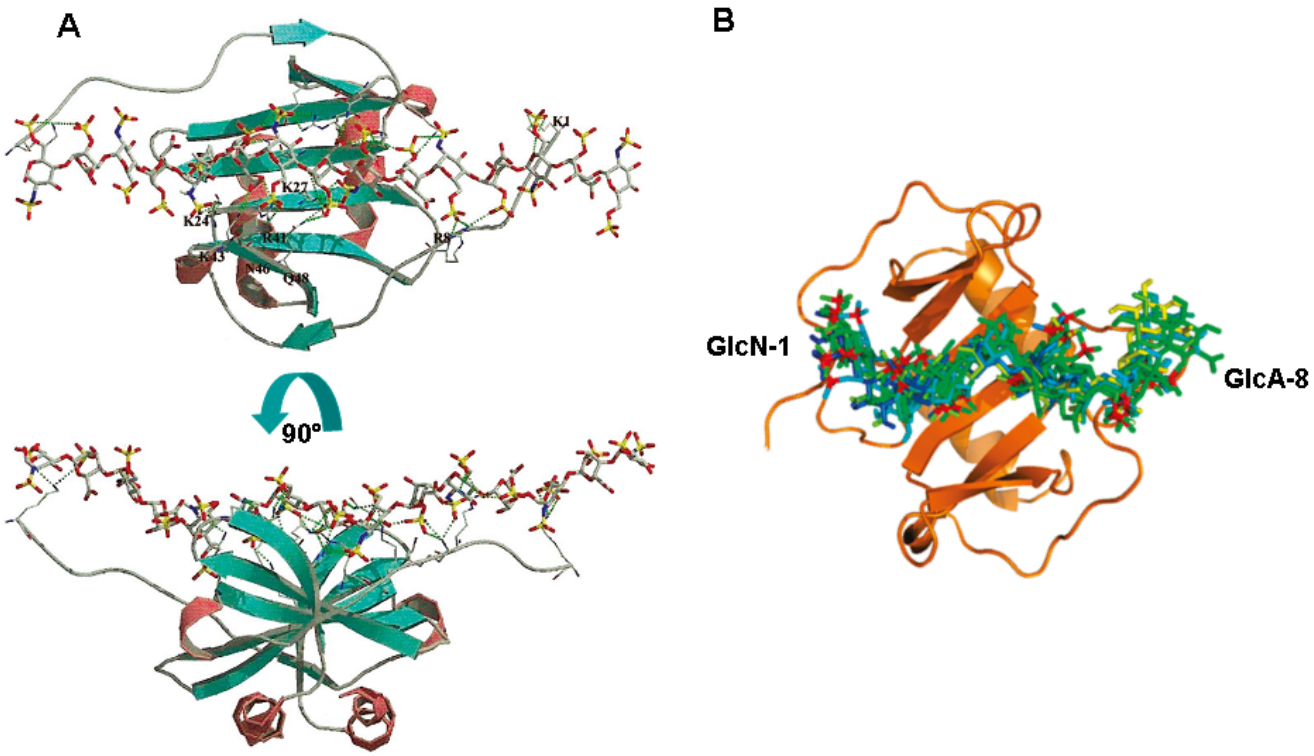


Figure 3.4 Model for the interaction between dimeric CXCL12α and an oligosaccharide (A) The CXCL12α is represented as a ribbon and the heparin oligosaccharide as well as the basic amino acids involved in the interaction are represented as sticks. Taken from (Sadir, Baleux et al. 2001). (B) Superimposition of 10 structures of the ^{13}C labelled octasaccharide onto one CXCL12α ribbon structure. Taken from (Laguri, Sapay et al. 2010).

3.4.6 Liaison with CXCR4 (Proposed Model)

The receptor binding domain is located in the N-terminus for almost all chemokines (Clark-Lewis, Schumacher et al. 1991; Clark-Lewis, Kim et al. 1995). The first proposed model for binding and receptor activation is a "two site" model for signalling through the coreceptor (Crump, Gong et al. 1997). The chemokine core (RFFESH) is proposed to bind first to the CXCR4; this serves as the initial docking step, "site one". Then the N-Terminal residues of the CXCL12α bind to

the more hidden CXCR4 acidic pocket amongst the extracellular loops 2 or 3 within the coreceptor (signal trigger, "site two") (Figure 3.5). More precisely, extensive mutational, structural and functional studies have revealed that the receptor activation requires Lysine 1 and Proline 2 within the N-terminal region and N-loop residues (between the second cysteine and the 3_{10} helix) (Crump, Gong et al. 1997). The first two residues (Lys1 and Pro2) activate the receptor through binding to the transmembrane helices and this has been demonstrated by showing that deletion or modification of the N-termini results in chemokines that do not induce signalling (Gong and Clark-Lewis 1995; Hemmerich, Paavola et al. 1999; Jarnagin, Grunberger et al. 1999). Also, Skelton *et al.,* determined the structure of CXCL8 in complex with a CXCR1 peptide and showed how the N-terminus of the receptor binds CXCL8 in such as way that the chemokines' N-terminus is oriented towards the receptor helices for receptor activation (Skelton, Quan et al. 1999).

This then induces a conformational change within the CXCR4, allowing intracellular G-protein binding and downstream signalling (Clark-Lewis, Schumacher et al. 1991; Farrens, Altenbach et al. 1996; Crump, Gong et al. 1997; Prado, Suetomi et al. 2007).

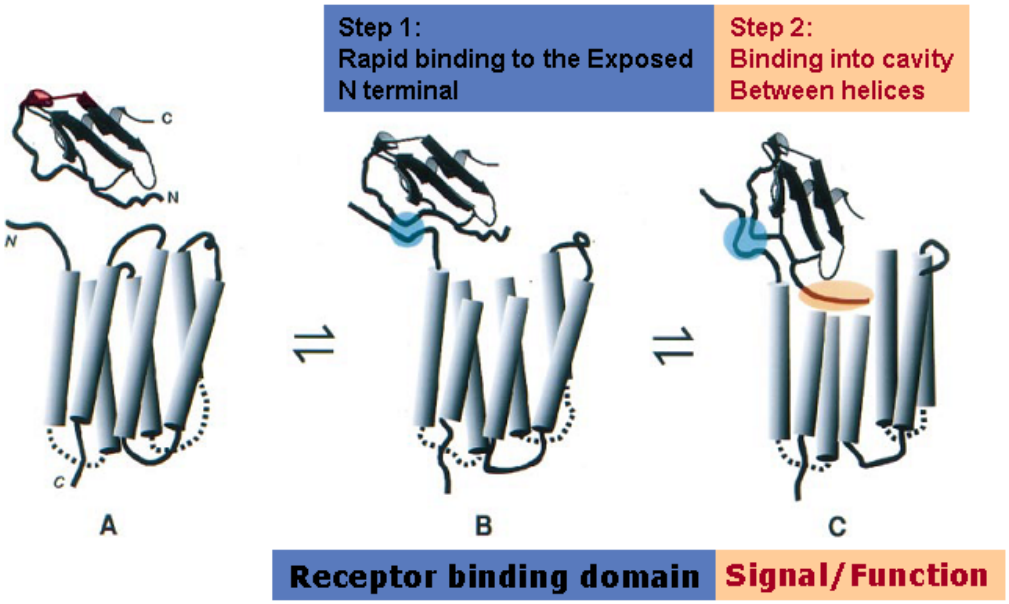


Figure 3.5 The "two site" binding model for the CXCL12α-CXCR4 interaction. Firstly, the N-loop of CXCL12α interacts with the CXCR4 N-terminus, this is followed by the N-terminus of CXCL12α binding the CXCR4 transmembrane region to trigger coreceptor activation (Crump, Gong et al. 1997).

Kofuku *et al.,* reported an interaction between full-length CXCR4 and CXCL12α and between CXCR4 and CXCL12α in the presence of the AMD3100 antagonist using NMR where they provide structural evidence supporting the theory that there are two independent interactions that occur between CXCL12α and CXCR4 (Kofuku, Yoshiura et al. 2009) – this supports the "two-site" binding model, and reveals many residues involved in addition to those proposed in the first model. This was the first time that an interaction was shown between the full-length

CXCR4 and CXCL12α. Using triple resonance experiments, ^{13}C- and ^{15}N-labelled MetCXCL12α was combined with CXCR4 expressed and purified from insect cells in the presence or absence of AMD3100. They showed that an extended surface on CXCL12α (consisting of the β-sheet, 50s loop and N-loop) first binds to the CXCR4 extracellular region which places the chemokine in a position to search the deeply buried binding pocket in the CXCR4 transmembrane region, where the CXCL12α N-terminus will bind - they call this the 'fly-casting' mechanism. They showed that AMD3100 could displace the CXCL12α N-terminus from the CXCR4 receptor without displacing the chemokine core domain (Kofuku, Yoshiura et al. 2009).

3.4.6.1 Characterisation of the CXCR4 N-terminus-CXCL12 complex

Previous studies have confirmed the involvement of the N-terminal of the receptor in chemokine binding. Many chemokine receptors have *O* or *N*-linked-glycosylations and/or are sulphated in their N-termini (CCR2, CCR5, CCR8, CXCR4, CX3CR1 …) and this post-translational modification increases the affinity of receptors for their generally basic ligands (Farzan, Mirzabekov et al. 1999; Farzan, Vasilieva et al. 2000; Bannert, Craig et al. 2001; Farzan, Babcock et al. 2002; Fong, Alam et al. 2002; Wang, Babcock et al. 2004). Tyrosine sulphation on chemokine coreceptors CCR2, CCR5 and CXCR4 increases their binding affinity with both chemokines and HIV-1 through their negatively charged sulphate groups (Farzan, Mirzabekov et al. 1999; Cormier, Persuh et al. 2000; Preobrazhensky, Dragan et al. 2000; Bannert, Craig et al. 2001; Farzan, Babcock et al. 2002).

In 2006, Veldkamp *et al.,* showed that a single sulphotyrosine-containing N-Terminal CXCR4 peptide has an increased affinity for CXCL12α (Veldkamp, Seibert et al. 2006). They showed using fluorescence polarisation experiments that the monomer-dimer equilibrium of CXCL12α is shifted towards a dimer in the presence of the sulphated peptide, as shown for chemokines in the presence of heparan sulphate (Veldkamp, Peterson et al. 2005). Veldkamp *et al.,* showed that the CXCR4 N-terminus bridges the CXCL12α dimer interface between the N-loop and the β3 strand and makes both polar and electrostatic contacts which are not observed with the monomer CXCL12α (Crump, Gong et al. 1997; Veldkamp, Seibert et al. 2008). The CXCL12α side chains Val18, Arg47 and Val49 make Nuclear Overhauser Effect (NOE) correlations with the sTyr21 ring of CXCR4 showing that the sulphotyrosine is only 5 Å away from these residues (Figure 3.6).

It has been suggested that the post-translational modification of the N-terminus of CXCR4 (tyrosine sulphation) contributes to the high affinity binding and recognition of CXCL12α (Farzan, Mirzabekov et al. 1999). In 2008, Veldkamp *et al.,* showed that CXCL12α dimerises in the presence of the first 38 amino acid residues of the N-terminal of CXCR4, containing 3 sulphotyrosines (positions 7, 12, 21) (Veldkamp, Seibert et al. 2008) (Figure 3.6 A).

Seibert *et al.,* 2008 found that CXCL12α, has an augmented binding affinity for CXCR4 (N-terminal residues1-38) with the increasing number of sulphated tyrosines, and this suggests that there is a potential physiological role for the

sulphation of the three tyrosine residues at the N terminus of CXCR4 (Seibert, Veldkamp et al. 2008). The structure resolution of CXCR4 has provided new insights and brought forward new questions regarding the interactions between CXCR4 and CXCL12α (Wu, Chien et al. 2010).

The structures of CXCR4 were determined as complexes with an antagonist small molecule (IT1t) and a cyclic peptide (CVX15) (Wu, Chien et al. 2010). The receptor crystallized as a homodimer in all five structures which suggests that there could be multiple binding configurations for CXCL12α and CXCR4. In the article, they propose three different binding models for CXCR4 and CXCL12α; either 1:1, 1:2 or 2:2 ligand:receptor complexes are feasible. Either monomeric CXCL12α binds monomeric CXCR4, or dimeric CXCR4 binds monomeric CXCL12α or dimeric CXCR4 binds dimeric CXCL12α. In the case of the 1:2 configuration, CXCL12α could bind one receptor with its core domain and *in trans* bind into the trans membrane pocket and activate the neighbouring receptor. The neighbouring coreceptor could be CXCR4 (homodimer) or CXCR7 (heterodimer), however, this is speculative.

Importantly, the non-structured N-terminus of CXCR4 was not present in the crystal structures and thus there are still speculations as to how the ligand binds CXCR4 in the presence of the N terminus. Molecular modelling studies and NMR studies by Veldkamp *et al.,* have predicted the orientation and stoichiometry of the chemokine binding to the N-terminal of CXCR4. Veldkamp *et al.,* proposes a CXCL12α dimer binding two N-terminal CXCR4 peptides, while Wu *et al.,* proposes three models, two of which constitute a CXCL12α monomer which binds either a monomer of CXCR4 or a homodimer of CXCR4 (Veldkamp, Seibert et al. 2008; Wu, Chien et al. 2010).Thus these two models are not in full agreement. However the exact understanding of this binding mechanism between the N-terminus of CXCR4 and the chemokine (in context of the entire coreceptor) is still not fully understood. The hyper-flexible and unstructured nature of the N-terminal of CXCR4, has rendered structural studies involving this region hugely challenging, nevertheless, all these models may be correct... the current CXCR4 structures are compatible with emerging concepts of signalling diversity induced by alternative binding modes of the ligands (Figure 3.6).

3.4.6.2 Signalling Activities of the CXCR4-CXCL12 complex

Recently, Drury *et al*., showed that CXCL12α monomers and dimers exert opposing effects on migration; migration in cell culture systems was detected with low concentrations of wild type CXCL12α, however, when low concentrations of constitutively dimeric CXCL12α was used or high concentrations of wild type CXCL12α, no migration was detected. Importantly, both the monomer and the dimer CXCL12α do activate their CXCR4 receptor to a lesser or greater extent (as shown by cell radioligand binding assays, intracellular calcium mobilization detection, CXCR4 internalisation etc) and they each activate discrete signalling profiles. In addition, the preferentially monomeric CXCL12α stimulated F-actin polymerisation mediated though β–arrestin and evoked a prolonged phosphorylation of ERK1/2. However, the constitutive dimer (disulphide bonds covalently locking two symmetric CXCL12α monomers [L36C/A65C]) (Veldkamp, Seibert et al. 2008) stimulated a transient increase in ERK1/2 with

minimal recruitment of β–arrestin and actin mobilisation. Could these differential migrational and signalling profiles of the monomer and dimer CXCL12α result from two different binding interactions between the chemokine and the receptor?

Drury *et al.,* proposes that when the N-terminal of CXCR4 is bound to a CXCL12α monomer, the first 10 residues are bound to the chemokine, however, when the N-terminal of CXCR4 is bound to the dimer, the first 10 residues of CXCL12α are more flexible, leading to the hypothesis that different oligomers of CXCL12α lead to different signalling pathways (Drury, Ziarek et al. 2011). However, this model is highly speculative due to the forced creation of the monomer and dimer CXCL12α forms and thus their plausibility is highly questionable.

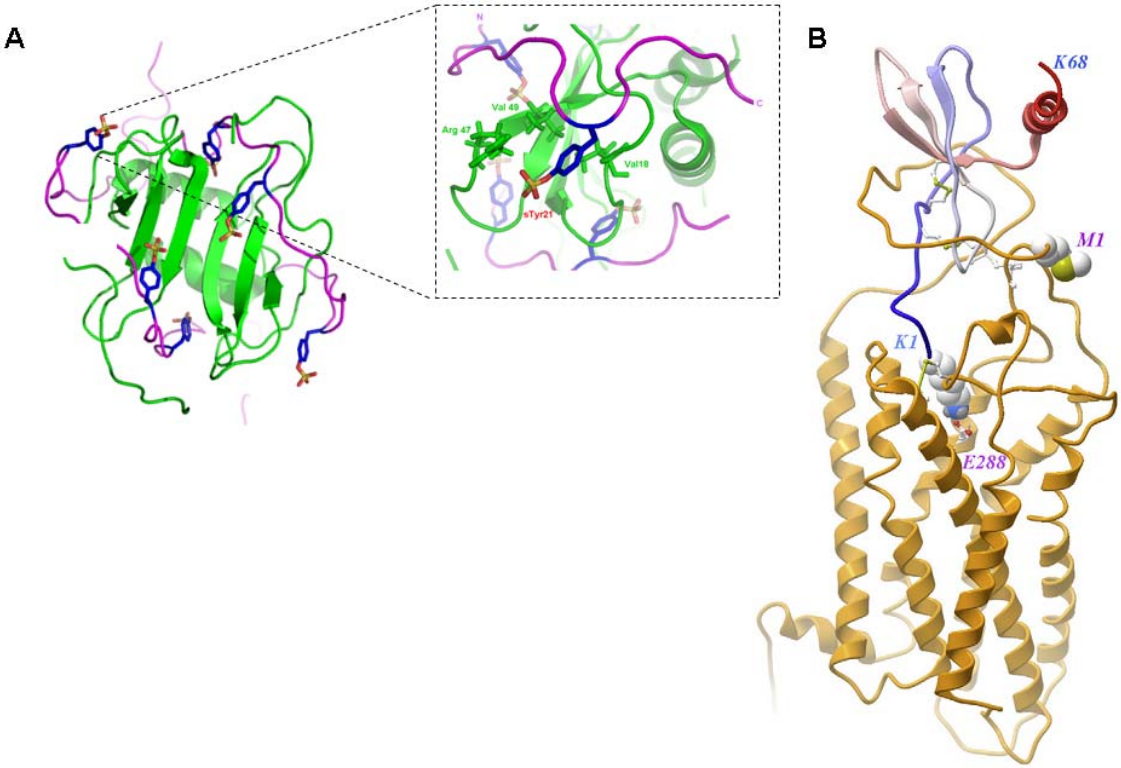


Figure 3.6 Comparison of proposed binding of (A) the 38aa sulphotyrosine peptide binding to a CXCL12α dimer revealed by NMR. The zoomed section shows the side chains CXCL12 residues Val18, Arg47 and Val49 that are involved in binding the sTyr21 of CXCR4 (Veldkamp, Seibert et al. 2008) and in (B) a monomer CXCL12α binding to CXCR4 revealed by molecular modelling (Salanga and Handel 2011).

3.4.7 Oligomerisation

The physiological relevance of the chemokine monomer-dimer equilibrium and the interaction between chemokines, GAGs and receptors is all not yet fully understood and highly controversial and thus will not be discussed in detail, however, it is important and thus is mentioned.

Most chemokines, including CXCL12α, tend to dimerize at high concentrations (Holmes, Consler et al. 2001; Gozansky, Louis et al. 2005; Veldkamp, Peterson et al. 2005; Baryshnikova and Sykes 2006; Veldkamp, Seibert et al. 2006). Monomeric variants of chemokines have been shown to be fully functional in chemotaxis assays *in vitro*, indicating that monomers are sufficient to activate receptors. However, several chemokines require oligomerisation *in vivo*, and all data suggest that this requirement is related to GAG-binding (as shown for MCP-1, MIP-1β, CXCL10 [IP-10] and RANTES) (Proudfoot, Handel et al. 2003; Campanella, Grimm et al. 2006).

It has been shown that receptors oligomerise (homodimers [CXCR4:CXCR4] and heterodimers [CXCR4:CXCR7]) and chemokines oligomerise (in the presence of GAGs and the N-terminal of CXCR4). Chemokine oligomers, including CXCL12α, appear to be functional and to induce alternative signalling responses, such as cellular activation or signals to halt migration (Veldkamp, Seibert et al. 2008), which give rise to the concept that these complexes dynamically change their stoichiometries and structures as part of their functional regulation. Further studies are required to elucidate the functional role of chemokine monomers and oligomers and the regulation of the equilibrium between the different forms and their different physiological roles.

3.4.8 CXCL12γ

Not only are there many unanswered questions pertaining to the regulation and specificity of CXCL12α signalling through CXCR4 and the role played by cell-surface and cell-free GAG oligosaccharides, but there are many unanswered questions about CXCL12γ too.

The mRNA of CXCL12γ was first identified in the heart of rats (Gleichmann, Gillen et al. 2000) and the structure-function relationships of this isoform are still not fully understood. Santiago *et al.,* have recently shown that CXCL12γ accumulates at the endothelium and on dendritic cells which are two important interfaces for T-cell recruitment and activation (Santiago, Izquierdo et al. 2011).

CXCL12γ is an alternative splice variant of CXCL12. Of the 98 amino acid residues which comprise CXCL12γ, the first 68 residues resemble a three dimensional structure which is closely related to that of the α isoform. However, the 30 residues at the C-terminal adopt an unstructured form which suggests a functional role due to its length, basic charge and mobility (Laguri, Sadir et al. 2007). Interestingly, 60% (18 basic residues) of the C-terminus of CXCL12γ are comprised of positively charged amino acids (lysine and arginine) and four HS binding motifs (BBXB) are found (Figure 3.3). This is why the C terminus of CXCL12γ has an extremely high affinity for negatively charged HS (K_D=0.9 nM versus 30nM for CXCL12α). This interaction is very stable, rendering the highest affinity interaction ever observed for any chemokine (Altenburg, Jin et al.; Laguri, Arenzana-Seisdedos et al. 2008; Rueda, Balabanian et al. 2008) (Figure 3.7). CXCL12γ is thus retained at the cell surface by the HS more so than the other isoforms. The C-terminal domain regulates the function of CXCL12γ as it is less active as compared to CXCL12α in terms of chemoattraction (Rueda, Balabanian et al. 2008), however, it broadens the GAG spectrum to which the chemokine can

bind. Rueda and colleagues showed that CXCL12γ signals through CXCR4 on lymphoid T cells, however, with a much lower agonist potency as compared to the CXCL12α isoform (Rueda, Balabanian et al. 2008). Interestingly, mutants of CXCL12γ that do not posses the BXBB HS binding motifs, show an increased affinity and activation of CXCR4 compared to the wild type (Laguri, Sadir et al. 2007; Rueda, Balabanian et al. 2008). The affinity of CXCL12γ for CXCR4 has never been calculated before.

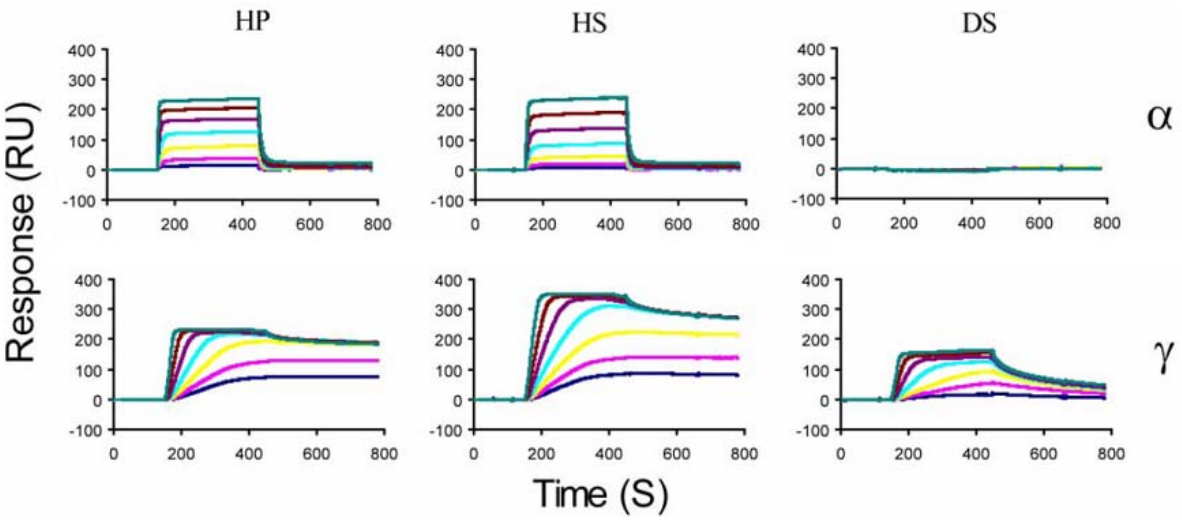


Figure 3.7 Analysis of CXCL12 binding to HP, HS and DS. SPR sensorgrams measured when CXCL12 was injected over HP, HS or DS activated sensorchips. The response in RU was recorded as a function of time (s) for CXCL12α (26 to 300 nM) and γ (2.6 to 30 nM). Adapted from (Laguri, Sadir et al. 2007).

The molecular and functional identity of this splice variant is heavily reliant on its unprecedented high affinity for GAGs and thus suggests that this chemokine may be under specific regulation by the HS found on the cell surface (Laguri, Sadir et al. 2007; Rueda, Balabanian et al. 2008). However, very little is known about the role played by this chemokine in homeostatic and pathological processes and its regulation by GAGs. Also, is it not known (and highly debatable) whether the inhibitory effect of soluble GAGs as seen for CXCL12α signalling can be applied to the CXCL12γ isoform.

Altenburg *et al.,* showed that CXCL12γ was the most potent at blocking CXCR4-tropic HIV-1 (Altenburg, Jin et al. 2010). The point in common between the anionic GAG fragments and the N-terminus of CXCR4 is the negatively charged residues; the sulphate groups along the oligosaccharide and the sulphated tyrosines respectively. CXCL12γ has a much higher affinity for GAGs when compared to the other isoforms and thus may have a higher affinity for the N-terminus of CXCR4.

The binding interactions between gp120 and chemokines with their cognate receptors as well as their interaction and regulation by GAGs are intimately linked. Understanding the structural basis for these interactions will provide valuable insight into the designing of better inhibitors of these interactions.

Another binding interaction that is highly speculative, is that between CXCL12 (α and γ) and CXCR7. There is only one tyrosine in the N-terminal of CXCR7, and the literature to date does not mention that this tyrosine is sulphated. From the work performed by Rueda *et al.,* in 2008, and as mentioned above, CXCL12α and CXCL12γ bind in an identical manner to CXCR7 (Rueda, Balabanian et al. 2008), which is clearly not the case for CXCR4. We can infer from these results that perhaps CXCL12γ binds to CXCR7 in a similar fashion to that of CXCL12α. It would thus be interesting to measure the signalling activity produced through CXCL12γ binding to CXCR7 and compare it to that of CXCL12α signalling through CXCR7. Also, with the recent discovery of CXCR7:CXCR4 heterodimers, one could imagine a scenario where the chemokine (for example CXCL12γ), binds the CXCR4 N-terminal via its elongated basic C-terminal and is able to reach to and signal through the adjacent CXCR7 chemokine. A similar scenario could be imagined for the CXCR4:CXCR4 homodimer; whereby a monomer CXCL12γ binds to the N-terminal of one CXCR4, and due to its heightened flexibility, is able to reach to the active site on the adjacent CXCR4 and induce a signal thorugh the adjacent CXCR4, while being tethered to the first CXCR4. The same situation could occur with CXCL12α, whereby it binds to one coreceptor (CXCR4 or CXCR7) and signals through the adjacent coreceptor in the dimer (CXCR4); this has already been proposed by Wu *et al.,* (Wu, Chien et al. 2010). In order to confirm these hypotheses, much futher experimentation is required.

Chapter 4: The Objectives of This Project

4.1 Les objectifs du projet (sommarie en français)

Au cours des 30 dernières années depuis la découverte du VIH-1, d'importants efforts ont été consacrés à la lutte contre ce virus. Malgré l'existence de nombreux anti rétroviraux, leur toxicité ainsi que le développement de virus résistants aux médicaments exigent l'emploi de stratégies d'attaques plus efficaces et innovantes. Une approche prometteuse et récente consiste à cibler l'entrée virale, et aujourd'hui, deux inhibiteurs d'entrée destinés au traitement ont déjà été approuvés par la Food and Drug Administration (FDA) et l'Agence européenne des médicaments (EMEA). Cependant, il existe déjà une résistance décelable ainsi que des effets secondaires indésirables associés à ces deux nouveaux médicaments. L'objectif global de ce projet consistait à mettre en place une plate-forme (de criblage), par laquelle différentes molécules pourraient être testées pour leur capacité à inhiber la liaison de la glycoprotéine de l'enveloppe virale (la gp120) à ses ligands de la surface cellulaire : les récepteurs couplés aux protéines G (GPCRs: CXCR4 et CCR5), le CD4 et les glycosaminoglycanes (GAG), inhibant ainsi l'entrée virale.

Des anticorps (17b, E51, 48d etc) ont été utilisés en tant que « mimes de corécepteurs » dans de nombreuses études, car ils lient la région cryptique de la gp120 qui n'est exposée qu'une fois que CD4 est déjà lié à l'enveloppe, c'est-à-dire les sites induit par CD4 (CD4i). L'utilisation de tels anticorps est moins fastidieuse et plus simple que l'utilisation de corécepteurs natifs, en raison des complications associées à la manipulation des protéines membranaires. Toutefois, l'utilisation d'anticorps en tant que mime de corécepteurs est problématique car l'anticorps n'est qu'un mime *partiel* et ne représente qu'une partie des épitopes qui sont réellement impliqués dans la liaison au corécepteur natif.

• C'est pour cette raison nous voulions mettre en place un système d'interaction qui permette la capture des corécepteurs natifs et leur utilisation dans une analyse d'interaction.

Traditionnellement, l'interaction gp120-corécepteur a été analysée avec des gp120 marquées ainsi que des cellules entières. Par conséquent, cette approche peut entrainer la liaison de gp120 à de nombreuses molécules à la surface des cellules. De tels systèmes sont donc mal adaptés aux tests de criblage car l'interaction gp120-corécepteur n'est pas isolée de la cellule entière. L'utilisation de corécepteurs isolés/purifiés, où la liaison gp120-CCR5 est détectée, est très peu abordée dans la littérature (Babcock, Mirzabekov et al 2001;.. Navratilova, Sodroski et al 2005). De surcroit, jusqu'à présent, l'affinité de l'interaction gp120-CXCR4 avec des corécepteurs CXCR4 isolés n'a jamais été détectée.

Les exemples de l'utilisation des protéines membranaires dans le contexte de la résonance plasmonique de surface sont aussi très rares dans la littérature. Ceci s'explique par l'extrême complexité des composants du tampon nécessaires pour maintenir la fonctionnalité de ces grandes protéines hydrophobes. L'un des

principaux objectifs de ce travail consistait à mettre en place et à utiliser cette technique dans le laboratoire. La mise en place de la méthode, qui consiste à solubiliser les corécepteurs et les immobiliser à une surface solide, tout en conservant leur fonctionnalité, représente une grande partie de notre travail, et constitue la principale évolution technique réalisée dans le cadre de cette thèse.

Un tel système d'interaction mets à profit l'interaction de forte affinité entre l'étiquette C-terminal du corécepteur CXCR4 et l'anticorps immobilisé 1D4 à la surface BIAcore. Le récepteur solubilisé est à la fois purifié et concentré sur la surface du capteur. Ceci est réalisé en présence d'un tampon contenant des lipides et des détergents qui pour garder la structure native des corecepteurs (GPCRs).

Afin de valider la fonctionnalité ce système d'interaction avec des corécepteurs solubilisés, nous avons d'abord utilisé le ligand naturel de CXCR4, CXCL12α / SDF1α. Des anticorps sensibles à la conformation de leur cible ont été utilisés afin de vérifier si nos corécepteurs solubilisés fonctionnaient ou pas, et si, en utilisant des techniques alternatives, leur interaction reflète les données cinétiques comparables aux études précédentes dans la littérature.

• Nous avons donc utilisé ce nouveau test d'interaction biochimique pour comparer la liaison de CXCL12α avec celle de CXCL12γ et leur partenaire CXCR4 [**Chapitre 5**].

• Une fois que la fonctionnalité du système a été validée, notre but ultime consistait à utiliser la plate-forme pour cribler plusieurs banques d'inhibiteurs d'entrée qui empêcheraient la liaison des complexes gp120-CD4 aux corécepteurs solubilisés et immobilisés [**Chapitre 6**].

En utilisant cette technique de résonance plasmonique, les corécepteurs solubilisés conservent leur fonctionnalité car des données cinétiques peuvent être déterminées entre les corécepteurs et leurs ligands respectifs. Cette technique met en évidence des informations sur les mécanismes de liaison des interactions spécifiques entre les GPCRs et leurs ligands, et fournit une plate-forme pour le criblage des antagonistes moléculaires. Ce système a été utilisé pour cribler diverses molécules inhibitrices de l'entrée du VIH-1. En plus de tester des molécules inhibitrices issues de nos programmes de recherche, nous nous sommes également servis de cette plate-forme pour cribler la capacité des petites molécules inhibitrices d'une entreprise commerciale à inhiber/empêcher l'entrée du VIH-1.

4.2 Objectives

During the last 30 years since the discovery of HIV-1, enormous efforts have been devoted to combating this virus. Many anti-retrovirals exist, however due to their toxicity and the development of drug resistant viruses, novel and more effective attacking strategies need to be employed. Targeting viral entry is a promising and recent approach and today, two entry inhibitors have been already approved by the FDA and EMEA for treatment. However, there is already detectable resistance and undesirable secondary effects associated with these two new drugs. The overall aim of this project was to set up a (screening) platform, whereby different molecules could be tested for their ability to inhibit the viral envelope glycoprotein, gp120, from binding to its ligands on the host cell surface, G-protein coupled receptors (GPCRs: CXCR4 and CCR5), CD4 and glycosaminoglycans (GAGs) respectively – thereby inhibiting viral entry.

Antibodies (17b, E51, 48d etc) have been used as 'coreceptor mimics' in many studies, as they bind the cryptic region on gp120 that is exposed only once CD4 has already been bound to the envelope, the CD4 induced site (CD4i). The use of such antibodies is less tedious and simpler than using native full-length coreceptors, due to the complications associated with manipulating membrane proteins. However, the draw-back to using antibodies as coreceptor mimics is that the antibody is only a partial mimic and does not fully represent all the epitopes that are actually involved in native coreceptor binding.

- It was for this reason desirable to set up an interaction system that enabled the native full-length coreceptor to be captured and used in a binding interaction analysis.

The gp120-coreceptor interaction has been traditionally analysed with labelled gp120 and whole cells; thus this approach could lead to gp120 binding to many cell-surface molecules. Such systems are poorly adapted for screening purposes as the gp120-coreceptor interaction is not isolated from the whole cell. The use of isolated/purified coreceptors, has been reported in very few papers (Babcock, Mirzabekov et al. 2001; Navratilova, Sodroski et al. 2005) where gp120-CCR5 binding has been detected, but up until now, the affinity of the gp120-CXCR4 interaction has never been detected with isolated CXCR4 coreceptors.

Reports of the use of membrane proteins in the context of surface plasmon resonance is very rare in the literature due to the extreme complexity of buffer components required to maintain the functionality of these large hydrophobic proteins. A major objective of this work was to set up and use this technique in the laboratory. Setting up the method (solubilising the coreceptors and immobilizing them on the solid surface, while retaining their functionality) represents a large part of our work and is the main technical development realized in the context of this thesis.

Such an interaction system takes advantage of the high affinity interaction between the C-terminal tag on the CXCR4 coreceptor and the immobilized 1D4 antibody on the biacore surface. The solubilized receptor is both purified and concentrated onto the sensor surface. This is performed in the presence of a

lipid/detergent containing buffer that mimics the bilayer where the coreceptors are naturally found.

In order to validate the functionality of the newly set-up solubilized coreceptor interaction system, we first used the natural ligands of CXCR4, CXCL12α /SDF1α. Conformationally binding-sensitive antibodies were used, as tools to verify whether our solubilized coreceptors were functional or not and if their interaction reflected comparable kinetic data to previous studies in literature using alternative techniques.

- Therefore, we made use of this new biochemical interaction assay to compare the binding of CXCL12α with that of CXCL12γ and their CXCR4 partner **[Chapter 5]**.
- Once the functionality of the system had been validated, our final endeavour was to use the platform to screen several banks of entry inhibitors which prevented the gp120-CD4 complex from binding to the solubilized immobilized coreceptors **[Chapter 6]**.

Solubilized coreceptors retain their functionality, using this surface plasmon technique, whereby kinetic data can be determined between coreceptors and their respective ligands. This technique elucidates information on the binding mechanisms of specific interactions between GPCRs and their ligands and provides a platform to screen for molecular antagonists. This system was used to screen for various inhibitory molecules of HIV-1 entry; not only were inhibitory molecules tested from collaborators but this platform was also used to screen small entry inhibitor molecules from a commercial company.

B: EXPERIMENTAL WORK

Chapter 5: GAGs differently effect the liaison of CXCL12α and CXCL12γ with CXCR4

5.1 L'héparane sulfate régule de façon différentielle la liaison de CXCL12 α et γ avec CXCR4 (sommaire en français)

L'héparane sulfate (HS), est apparu comme un régulateur clé de nombreux processus biologiques fondamentaux. Beaucoup de protéines, parmi lesquelles les chimiokines se lient à HS et cette interaction est fonctionnellement importante. CXCL12, une chimiokine dont les nombreuses fonctions biologiques sont médiées par un récepteur particulier couplé aux protéines G (CXCR4), existe dans six différentes isoformes, les plus étudiées étant CXCL12α. Des études antérieures ont montré que CXCL12α interagit avec HS avec une affinité de 50 nM, tandis que l'isoforme CXCL12γ affiche une affinité inhabituellement élevée pour cet GAG (Kd = 0,9 nM) (Laguri, Sadir et al. 2007). La base de cette différence a été attribuée à un long stretch de résidus basiques qui caractérisent le domaine C-terminal de CXCL12γ et absent dans CXCL12α. Des études *in vivo* ont montré que la liaison avec HS permet à CXCL12γ de promouvoir les activités biologiques avec beaucoup plus d'efficacité par rapport à CXCL12α. Nous avons comparé l'interaction de ces deux isoformes avec CXCR4, et étudié le rôle des HS dans cette liaison. Ici nous avons utilisé la technologie SPR dans lequel CXCR4 solubilisé a été capturé dans un environnement lipidique / détergent sur une surface de biocapteur afin de mesurer la liaison de CXCL12α et CXCL12γ en temps réel. Les affinités obtenues (Kd de 13 et 0,7 nM pour les isoformes α et γ, respectivement) sont en corrélation avec les valeurs déterminées pour les récepteurs membranaires dans d'autres études (pour CXCL12α). La préincubation de CXCL12α avec HS n'a pas modifié sa liaison à CXCR4, mais a fortement diminué celle de la de l'isoforme CXCL12γ, un point qui a été confirmé par cytométrie de flux sur des des cellules. Cela suggère que la partie C-terminale de CXCL12γ contribue à la liaison à CXCR4, vraisemblablement en interagissant avec l'extrémité N-terminale sulfatée de CXCR4 (les tyrosines 7, 12 et 21 dans le domaine N-terminal sont sulfatées). Ceci a été confirmé par une expérience de titration en utilisant des approches de RMN. Lorsque la protéine recombinante CXCL12γ ^{15}N marquée a été titrée avec des peptides sulfatés du N-terminus de CXCR4, les données suggèrent que des sulfotyrosines dans le domaine N-terminal de CXCR4 renforcent l'interaction avec CXCL12γ, et que le C-terminal de la chimiokine CXCL12γ est responsable de l'augmentation de l'affinité avec le corécepteur. L'analyse de phosphorylation de ERK induite par CXCL12α et CXCL12γ montre que les deux isoformes activent différemment la cascade de signalisation, suggérant un rôle du domaine C-terminal de la chimiokine. Ensemble, ces données montrent que le C-terminal basique et allongé de CXCL12γ interagit à la fois avec les HS et la séquence sulfatée N-terminale de CXCR4 et nous proposons une éventuelle régulation de l'activité de l'isoforme CXCL12γ par HS.

5.2 Introduction

An assay that permits the isolation and immobilization of functional G protein coupled coreceptors / GPCRs is extremely valuable as it permits the analysis of very complex molecular interactions that occur at the cell membrane so that they can be studied with the least amount of other or non-specific binding partners present. Not only is it of paramount importance to search for an effective HIV-1 entry inhibitor that can inhibit both CCR5 and CXCR4 tropic HIV-1 entry, but the mechanisms of chemokines binding to their coreceptors are not fully understood and such an assay can help understand these complicated binding mechanisms as well as assess the roles played by glycosaminoglycans in these interactions, which is also poorly understood.

Our collaborators (Francoise Baleux, Institut Pasteur) synthesized the different isoforms of CXCL12 (α, γ and mutants used in this study). Using this protein as a ligand for the solubilized CXCR4 on the biacore is strategic for two reasons: i) firstly it is a relatively small (8-10 kDa), soluble protein that will render information on whether or not the solubilized CXCR4 coreceptors are functional and ii) since our collaborators synthesize these proteins, they are available in large, homogeneous amounts, as well as mutants of these proteins.

Membrane proteins are notoriously difficult to manipulate once they have been removed from their natural environment. Their large hydrophobic domains required the presence of a delicate balance of certain lipids and detergents to retain their functional three-dimensional structure once they have been extracted from the cell membrane. Failure to solubilize the coreceptors in the correct cocktail of lipids and detergents, results in denatured or only partially folded coreceptors which are not recognized by their ligands and thus are not functional and consequently irrelevant in binding studies. Very few groups in the world endeavour to solubilize and capture GPCRs on a biacore surface for binding studies (Navratilova, Sodroski et al. 2005). Some studies perform binding interactions on whole cells using labelled ligands (Doranz, Baik et al. 1999), however, this can lead to non-specific binding of the ligands to the cell surface molecules (such as glycosaminoglycans). Other groups incorporate the solubilized GPCRs into proteoliposomes for binding studies (Babcock, Mirzabekov et al. 2001; Zhukovsky, Basmaciogullari et al. 2010). Such work with proteoliposomes requires labelling of the ligand which could alter the binding properties of the ligand. Thus, the approach of solubilising and immobilizing solubilized GPCRs on a static surface allows for the least amount of non-specific binding, no requirement for ligand labelling and the reaction can be followed in real-time.

In the context of the objectives of this work, in order to set up an interaction system whereby the binding events between immobilized GPCRs (CXCR4 and CCR5) and their partners (gp120 and CD4) can be monitored and the inhibitory capacity of the mCD4-HS_{12} evaluated and further improved, a binding assay was first set up between CXCR4 and CXCL12α, to verify the integrity of the immobilized coreceptors. Below, is a cartoon representation of the immobilization of the solubilized coreceptors through their interaction with the 1D4 antibody which is coupled to the dextran surface by amine coupling chemistry (see

section 8.10.5.2). Various proteins and antibodies are seen interacting with the solubilized coreceptors and these are representations of what we have studied using the biacore. In every experiment, a reference cell was used whose binding response data was subtracted from the test cell so as to remove any non-specific signal if any and to correct for bulk effect associated to ligand injection (Figure 5.1).

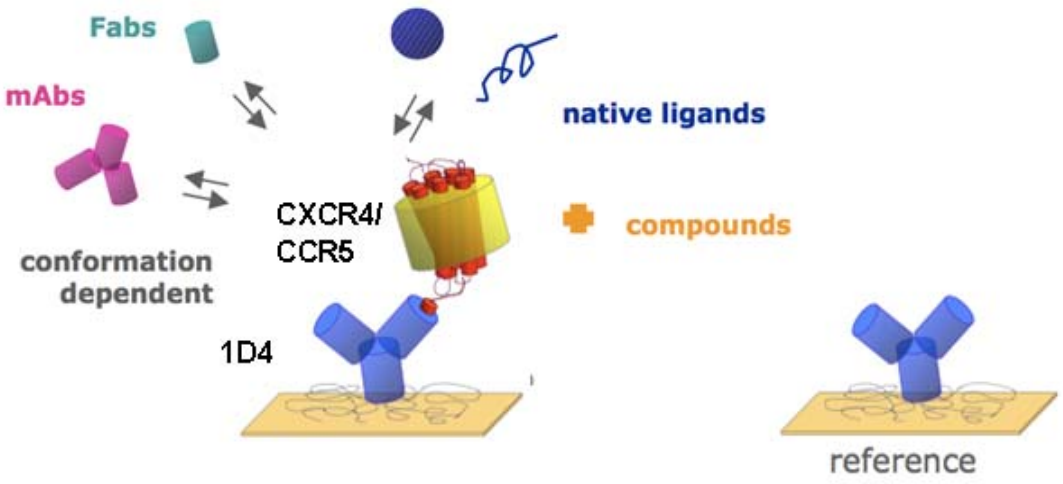


Figure 5.1 Cartoon of the capture of C9 tagged GPCRs through their interaction with the 1D4 immobilized antibody and the binding of conformation specific antibodies, ligands and compounds. Adapted from (Navratilova, Dioszegi et al. 2006).

5.3 Results

5.3.1 Preparation and control of cells expressing CXCR4

The canine thymocytes (Cf2Th cells) that were used in this assay are adherent cells that have been stably transfected with the human clones of either CCR5 (Cf2Th.CCR5) or CXCR4 (Cf2Th.CXCR4) and thus constitutively express high levels (0.5 - 1.0 x 10^6) of human CCR5 and CXCR4 coreceptors per cell respectively. The clones were originally made by (Dr. Tajib Mirzabekov and Dr. Joseph Sodroski) and a C-terminal tag (TETSQVAPA [C9]) was incorporated into the sequence (Mirzabekov, Bannert et al. 1999). The C9 tag has a high affinity for the 1D4 antibody and thus this tag-antibody recognition system allows for an efficient strategy to purify the coreceptors from crude membrane extractions. These cells were cultured in standard DMEM media supplemented with serum and antibiotics as previously described (Mirzabekov, Bannert et al. 1999). The cells were serially passaged five times before harvesting. On the day of a coreceptor extraction, the cells were washed in PBS, and detached from the flask with versene (0.48 mM EDTA in phosphate-buffered saline). The Cf2Th cells grew relatively very fast (confluent within 2-3 days) and detached from the culture flasks easily in the presence of versene.

Before the interaction system was set up using surface plasmon resonance (SPR), we initially verified the expression of the GPCRs on the surface of the Cf2Th cells using flow cell cytometry. The conformationally sensitive antibodies 2D7 (Lee, Sharron et al. 1999; Khurana, Kennedy et al. 2005) and 12G5 (Baribaud, Edwards et al. 2001) were systematically used to detect CCR5 and CXCR4 respectively on the cells using flow cell cytometry analysis (Figure 5.2).

Briefly, 1 x 10^6 cells (expressing either CCR5 or CXCR4) were detached from the cell culture flasks and washed in PBS. They were then incubated with the primary antibodies (2D7 and 12G5-FITC respectively) for one hour at 4°C. The unbound antibodies were washed away three times in PBS and the secondary FITC-labelled antimouse antibody was used to detect the bound 2D7 to the cells. The 12G5 was coupled to FITC directly and did not require a secondary antibody. The FITC-labelled cells were fixed in 4% paraformaldehyde and detected in the flow cytometer. As can be seen from the intensity of the bound antibodies to the Cf2Th cells, the presence and integrity of the coreceptors was verified (Figure 5.2).

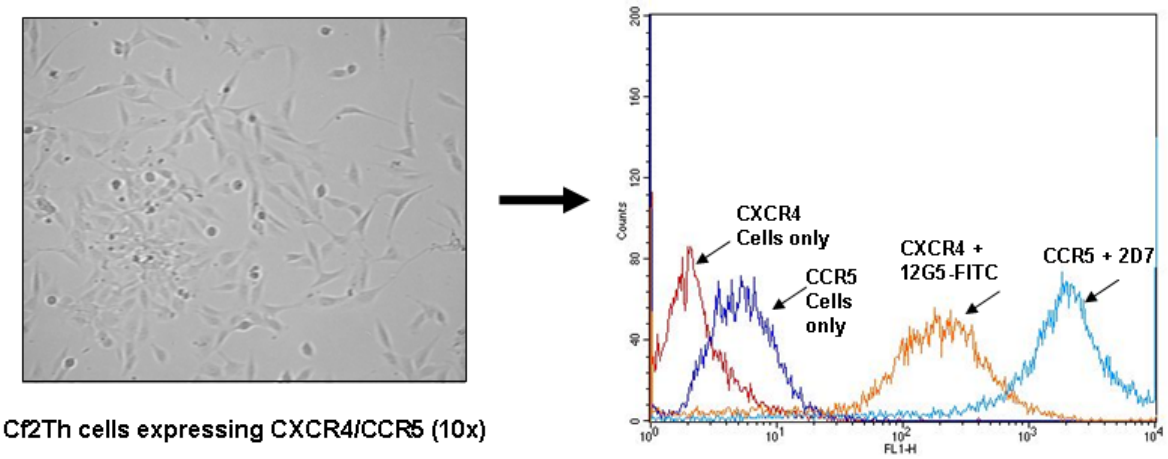


Figure 5.2 Cf2Th cells expressing either C-terminal C9 tagged CXCR4 or CCR5 (10x) and corresponding flow cytometric analysis depicting a positive stain for 12G5-FITC binding to CXCR4 expressing Cf2Th and 2D7 binding to CCR5 expressing Cf2Th cells.

5.3.2 Alternative methods to measure binding

Initially, we sought to develop a flow cell cytometry-based technique, whereby the binding of conformationally dependent antibodies to their coreceptors (e.g. 12G5 binding to CXCR4) was displaced in the presence of competing molecules (T134 or gp120-CD4) or binding was restored in the presence of 'inhibited' competing molecules (CXCL12α non-binding mutants or complexes of gp120-CD4 + inhibitor). For example, as shown in Figure 5.3, for both normal cells and cells that had been stored in 0.4 M sucrose (Sadir, Lortat-Jacob et al. 2000), 12G5-FITC binding was diminished in either the presence of non-labeled 12G5 or in the presence of T134, a CXCR4 antagonist (Xu, Tamamura et al. 1999). Thus techniques seemed feasible for the use of T134, however, in our hands we were unable to show diminished 12G5-FITC binding in the presence of competing molecules such as gp120.

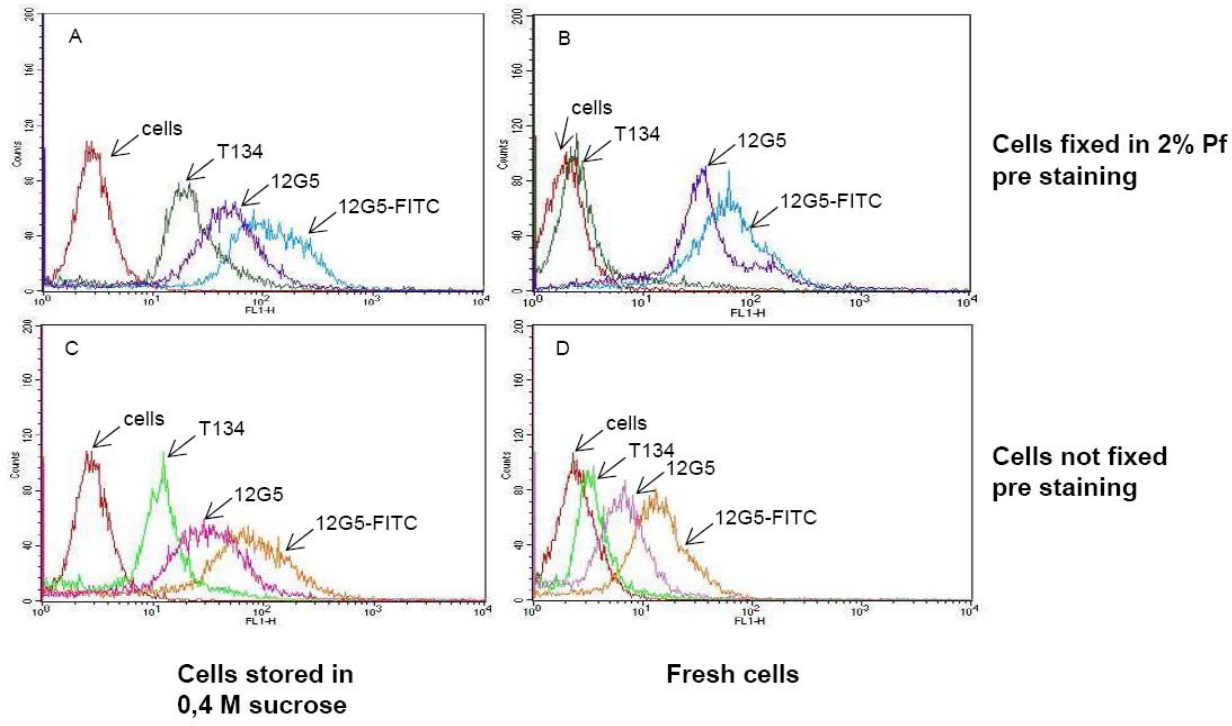


Figure 5.3 The upper panels show cells that have been treated with 2% paraformaldehyde (pf) before staining with the 12G5-FITC secondary antibody and the lower panels were not fixed in pf. Panels on the left were performed with cells stored in 0.4M sucrose and panels on the right are fresh cells. Unlabelled CXCR4 expressing cells (red), pre-incubation with 5µg/ml T134 (dark and light green), pre-incubation with 12G5 (purple and pink), and cells directly labelled with FITC-12G5 (blue and orange) are shown.

5.3.3 Solubilization of the CXCR4 membrane protein

Following this, we analysed whether solubilised coreceptors, despite the abrasive treatment to solubilise them from the cell membranes, retained their integrity and were still able to be detected by their ligands (12G5, CXCL12α). This would give us an indication whether or not the solubilization process would be suitable to capture functional coreceptors on the SPR surface for binding analysis.

Due to the hydrophobic nature of the coreceptors, combinations of lipids (DOPC : DOPS) and detergents (DDM, CHAPS and CHS) in different buffer compositions were tested to identify the conditions that retain the functionality of the solubilised coreceptors. To confirm the integrity of the immobilized coreceptors, antibodies and the CXCR4/CXCL12α interaction was analysed. The solubilization buffer and protocol used in this study was slightly adapted from that of Navratilova *et al.,* (Navratilova, Sodroski et al. 2005). Usually 5 x 10^6 cells per ml of solubilization solution were solubilized for the preparation of the membrane-derived coreceptors and this solubilization solution was tested for its ability to retain the functional integrity of the coreceptors during and after the solubilization.

5.3.4 SPR Analysis of solubilized CXCR4

The solubilized coreceptors were then captured onto a 1D4 immobilized chip surface. For this purpose, the 1D4 antibody surface was prepared as previously described (Navratilova, Sodroski et al. 2005). Briefly, the carboxylmethyl surface was activated with a 1:1 mixture of EDC/NHS which activated the carboxyl groups on the sensorchip surface (see section 8.10.5.2 for details). Following this, the 1D4 (anti-C9 tag) antibody was injected onto the sensorchip in a Na Acetate buffer with a pH of 4.2 (a buffer with a pH that is usually 1 unit down from the proteins' pI), so as to ensure that the antibody was protonated and thus with its overall positive charge will aid its attraction to the activated carboxyl surface. An injection of about 12 minutes at 5µl/min ensured that a sufficient (~7000 response units) amount of 1D4 was immobilized onto the surface. The remaining free activated carboxyl groups were blocked with a 10 minute injection of 1M ethanolamine pH 8.5. A representative antibody and coreceptor immobilization profile are shown in Figure 5.4 A. This 'ID4 surface' was the pre-requisite for capturing the solubilized coreceptors via their C-terminal tag (C9).

Once the ID4 surface was formed, the buffer was changed from the HEPES Buffered saline to the running buffer containing lipids and detergents. The solubilized coreceptors were injected in the latter buffer for a period of 30-60 minutes at a low flow rate (5µl/min) to ensure maximum coreceptor capture via the 1D4-C9 tag interaction over the ID4 surface (Figure 5.4B).

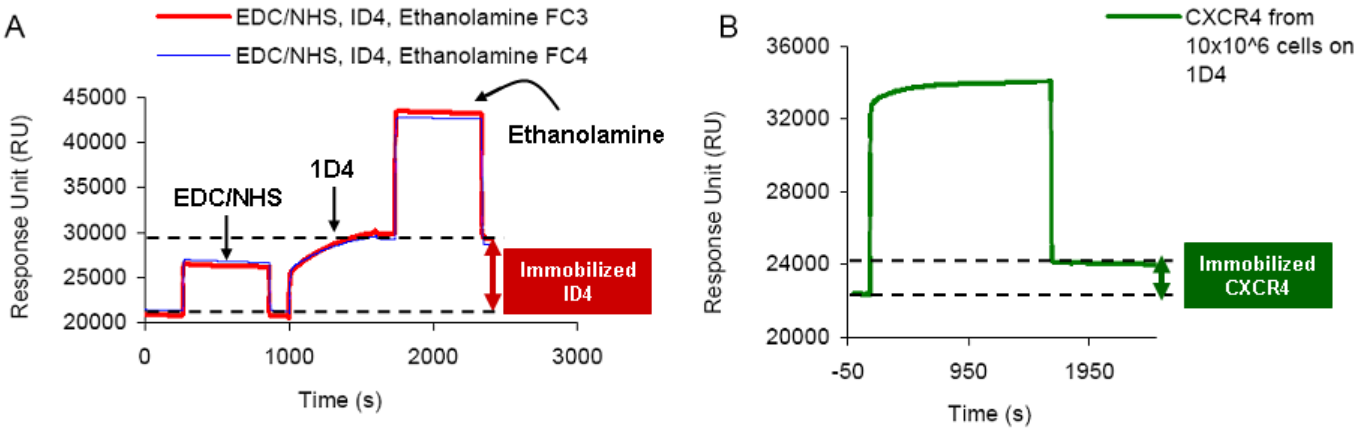


Figure 5.4 (A) Representative sensorgram of EDC/NHS injection, 200µg/ml 1D4 immobilization and ethanolamine blocking on the CM4 sensor chip surfaces to ~7000 ru of 1D4. (B) Representative sensorgram of coreceptor immobilization via the C9 tag -1D4 interaction to ~3000 ru.

Initially, a previously published buffer (Navratilova, Dioszegi et al. 2006) was used to analyse interactions between CXCR4 and its ligands (12G5, CXCL12α etc) using SPR, however, the binding curves were often not smooth and not reproducible. Thus we altered the buffer slightly and used a new composition, the Rebecca Rich buffer. Even further optimization was required to obtain reproducible binding data and we finally ended up with a completely unique buffer (*New buffer*) (Table 5). The interaction between between immobilized HS and CXCL12α has been reported previously in a standard aqueous buffer (10mM

HEPES pH 7.4, 150mM NaCl) (Amara, Lorthioir et al. 1999). In order to verify that our complex buffer constituents did not alter the binding properties of CXCL12α, we tested the binding of this classical and well-known SPR interaction, between CXCL12α and immobilized HS, in the presence of the *New buffer*. Equivalent binding responses between immobilized HS and CXCL12α in the *New buffer* were reproduced as compared to those observed by Amara *et al.,*. This verified that the complex buffer components do not alter CXCL12α's binding properties to its partner HS and thus we could proceed to perform further tests with CXCL12α in the presence of the *New buffer*.

Table 5. Summary of the different buffers tested during the optimization of the BIACORE running buffer. The final buffer used is the New buffer.

Classical Buffer	Navratilova Buffer (Navratilova, Dioszegi et al. 2006)	Rebecca Rich Buffer	*New Buffer* (This work)
50mM HEPES pH 7.4 150mM NaCl	50mM HEPES pH 7.0 150mM NaCl 1 mM $CaCl_2$ 5 mM $MgCl_2$ 0,1 % DOM 0,1 % CHAPS 0,02 % CHS 50 nM 7:3 DOPC :DOPS 0,1 mg/ml of BSA	50mM HEPES pH 7.0 150mM NaCl 5 µM $CaCl_2$ 1 µM $MgCl_2$ 0,1 % DDM 0,1 % CHAPS 0,02 % CHS 500 nM 7:3 DOPC :DOPS 0,2 mg/ml of BSA 5% glycerol 5 % PEG 8000 3% DMSO	50mM HEPES pH 7.0 150mM NaCl 5 µM $CaCl_2$ 1 µM $MgCl_2$ 0,1 % DOM 0,1 % CHAPS 0,02 % CHS 50 nM 7:3 DOPC :DOPS 0,1 mg/ml of BSA 5% glycerol 5 % PEG 8000

Since it was established that the *New buffer* retained the full functionality of the CXCL12α, the crude preparations of coreceptors in the *New buffer* described above were injected over the 1D4 immolbilized surface as described in Figure 5.4 (Test surface). In parallel, the negative surface was used, this surface contained only the 1D4 antibody and any non-specific binding between the CXCR4 ligands and the 1D4 antibody, were subtracted from the test surface for the final binding curve.

To confirm the integrity and functionality of the immobilized coreceptors, interactions between conformational antibodies and chemokine ligands were monitored. For this purpose, 25nM 12G5 was injected over the coreceptor and 1D4 (negative) surfaces. The mAb 12G5 recognises complex conformationally-dependant epitopes in the first and second extracellular loops of CXCR4 (requires an intact C28-C274 disulfide bond) and thus this antibody was used to asses the integrity and functionality of the immobilized coreceptors (Brelot, Heveker et al. 1999). The CXCR4 surface interacted with the 12G5 and to confirm that this interaction was specific, 1µM of T134, a small molecule CXCR4 inhibitor (14

amino acid peptide antagonist) was injected over the surface in the presence of 25nM 12G5. T134 was previously shown to inhibit 12G5 binding to CXCR4 expressing cells at a similar concentration (Xu, Tamamura et al. 1999). The T134 almost completely inhibited 12G5 binding, confirming that the 12G5/CXCR4 interaction was specific (Figure 5.5 A).

Now that we were convinced that the solubilized CXCR4 coreceptors were functional, through the specific binding of the 12G5 antibody, we then injected 50nM of CXCL12α over the CXCR4 surface and this chemokine gave rise to a typical binding sensorgram (association phase followed by a dissociation phase returning to the baseline after the end of the injection) (Figure 5.5 B). CXCL12α binding to CXCR4 has been described as a two step process; whereby the N-loop, as well as residues in the β-sheet and 50-s loop first make contact with the N-terminal of CXCR4, this is then followed by the first three residues in the chemokine N-terminal which reach deep into the trans-membrane region of CXCR4 for receptor activation (Crump, Gong et al. 1997; Kofuku, Yoshiura et al. 2009).

To confirm specificity of the CXCL12α/CXCR4 interaction, we injected two different mutants over the CXCR4 surface; CXCL12α 2-67 and CXCL12α 5-67. Each mutant was truncated within the receptor activation domain (the first KP residues). Thus, in agreement with the model, these residues are crucial to CXCR4 binding, without these residues, the chemokine binds less to the coreceptor (Figure 5.5 B). This further suggests that the solubilized captured coreceptors adopt a conformation that is very close to their native one.

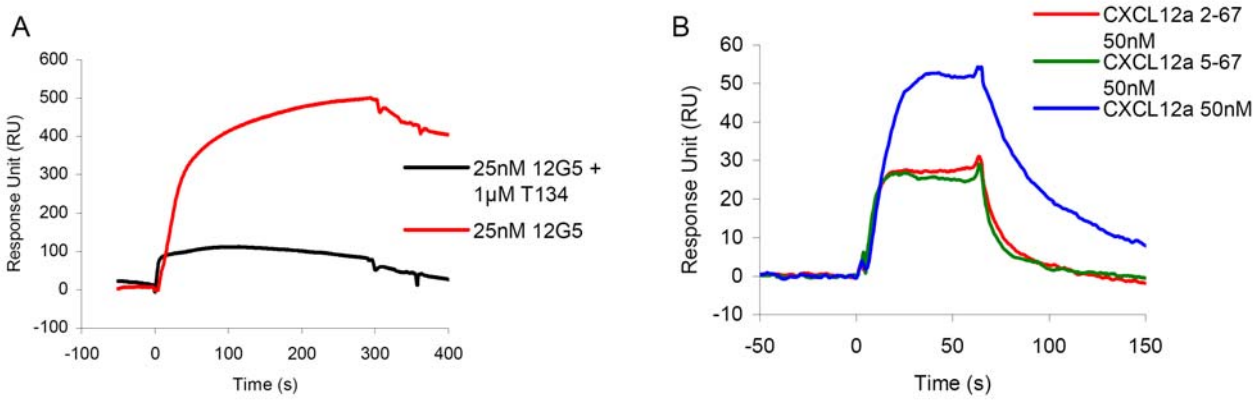


Figure 5.5 (A) 25nM 12G5 binding to immobilized CXCR4 in the absence (red curve) and presence (black curve) of 1μM T134 antagonist. (B) Native CXCL12α (50nM, blue curve), mutant CXCL12α 2-67 (red curve) and mutant CXCL12α 5-67 (green curve) binding to immobilized CXCR4.

Our collaborators have also synthesized the CXCL12γ isoform. As mentioned above, this is the longest CXCL12 isoform, consisting of the same first 68 amino acids found in CXCL12α, however, there are an additional 30 amino acids on its C-terminal which are the product of alternate splicing. This extended C-terminal contains three classic BBXB heparan sulphate binding domains. Whether or not the recently described CXCL12γ isoform and CXCL12α isoform interact in a similar fashion with their receptor, CXCR4 was then investigated. Dose response

experiments were performed for a range of concentrations (5 – 50nM) with both CXCL12α and CXCL12γ isoforms over the immobilized CXCR4 surfaces. Visual inspection of the curves (Figure 5.6 A) immediately illustrates that CXCL12α and CXCL12γ display remarkable differences both in the intensity of the binding curves and in the stability of the complex formed once injected over the CXCR4.

We fitted the responses for the CXCL12α /CXCR4 interaction to a 1:1 langmuir interaction model and the on rates (k_{on} or k_a) were calculated as $2.58 \times 10^6 \pm 5.9 \times 10^5$ $M^{-1}s^{-1}$ and off rates (k_{off} or k_d) calculated as $3.36 \times 10^{-2} \pm 5.9 \times 10^{-3}$ s^{-1}. The CXCL12α/CXCR4 was not very stable as seen by the rapid dissociation of the binding curves, however, the affinity (K_D) of this interaction was relatively high and calculated at 13 ± 1.6 nM. The value obtained for the affinity of CXCL12α/CXCR4 is in the same range as that reported by a similar technique (Navratilova, Dioszegi et al. 2006) and a ^{125}I-labelled CXCL12α binding assay (Crump, Gong et al. 1997) with membrane-associated receptor (Di Salvo, Koch et al. 2000) (Figure 5.6 A). Thus, this technique of measuring binding interactions of ligands to solubilized coreceptors is functional.

As for CXCL12α, we observed a concentration dependant binding response for CXCL12γ. The responses (in RU) were much stronger for this isoform compared to those of CXCL12α as can be seen from the binding curves (Figure 5.6 B).The dose response curves were fitted to a 1:1 interaction model with mass transfer because the binding was limited by diffusion. This means that the kinetic binding rate is significantly higher than the rate of transfer of analyte to the surface. In such a case, the association phase is slower due to the analyte not reaching the surface fast enough and the dissociation phase is also slowed down because the analyte is not transferred away fast enough from the surface and can thus rebind. Hence when there is a mass transfer limitation, a higher flow rate and lower surface density is recomended to increase the rate of transfer of analyte to the surface and decrease the surface binding capacity respectively.

When fitting the data, this mass transfer effect was taken into consideration. The calculated affinity is also higher as seen by the very slight dissociation of the chemokine at each concentration. The k_{on} for CXCL12γ was calculated as $k_{on} = 1.05 \times 10^7 \pm 1.2 \times 10^7$ $M^{-1}s^{-1}$ and dissociation rate constant was calculated as $k_{off} = 5.6 \times 10^{-3} \pm 5.3 \times 10^{-3}$ s^{-1}. The affinity was calculated as a ratio between the k_{on} and the k_{off} values; $K_D = 0.7 \pm 0.3$ nM, this is the first time an affinity has been calculated for this isoform (Figure 5.6 B). The k_{on} and the k_{off} values have been determined as a median of two independant experiments and such a large variation is seen with the standard deviations because this reaction happens very quickly, and such a high affinity reaction pushes the detection limits of the biacore apparatus. The on-rate is so fast that almost all of the dissociation is limited by mass transport and the flow rate cannot remove dissociating ligands away fast enough.

Interestingly, CXCL12α rapidly dissociated from the immobilized coreceptors while CXCL12γ formed tight complexes as seen in the case for the CXCL12γ chemokine binding to HP, HS and DS (Laguri, Sadir et al. 2007). Since the sole structural difference between CXCL12α and CXCL12γ is the extended basic C-Terminal of CXCL12γ, these results suggests the involvement of this extension in

CXCR4 binding and that this region may be responsible for the heightened affinity of CXCL12γ for CXCR4.

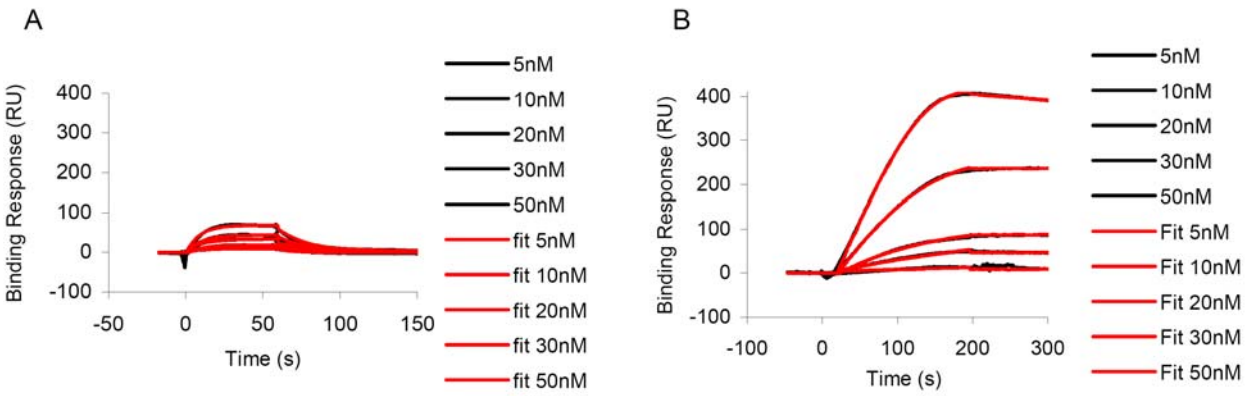


Figure 5.6 (A) Binding of a range of concentrations (from top to bottom) 50, 30, 20, 10, 5nM of CXCL12α or the same range of concentrations of CXCL12γ (B) over immobilized CXCR4. The black traces correspond to the experimental data and the red traces correspond to the fitted data using a 1:1 langmuir model (A) and a 1:1 Langmuir model with mass transfer (B).

5.3.5 The role played by GAGs

Laguri *et al.,* demonstrated that the γ isoform of CXCL12 displays an unprecedented high affinity for heparan sulphate (K_D = 0.9 nM). The unfolded 30 amino acid C-terminal tail of CXCL12γ distinguishes itself from the α isoform and thus it is this extended basic C-terminal of the chemokine that binds to HS. Previous studies have shown that the high affinity that CXCL12γ has for HS (Laguri, Arenzana-Seisdedos et al. 2008; Rueda, Balabanian et al. 2008) demonstrates a strong participation of the CXCL12γ C-terminal in the interaction. Advantage was taken of this high affinity interaction to investigate the importance of the CXCL12γ C-terminal tail and the possible role of GAGs played in regulating binding of the chemokine to its receptor.

We thus performed an assay where either CXCL12α or CXCL12γ was injected over a CXCR4 immobilized surface in the presence or absence of HP12 (dp 12 of heparin). A 12mer was chosen since this length was identified (using SPR techniques, confirmed by molecular modelling) for maximal binding capacity to CXCL12α (Sadir, Baleux et al. 2001; Sadir, Imberty et al. 2004).

As shown in Figure 5.7A, 50nM CXCL12α in the presence of 1µg/ml HP12 (~278 nM), shows no significant change compared to the binding between CXCL12α and CXCR4. This observation is consistent with the fact that the HS and the CXCR4 binding sites on CXCL12α do not overlap (Laguri, Arenzana-Seisdedos et al. 2008). However, when 50nM CXCL12γ is injected in the presence of 1µg/ml HP12, there is a 7 fold decrease in the steady state equilibrium compared to 50nM CXCL12γ binding to CXCR4 (Figure 5.7B). This suggests that in the presence of a 12mer oligosaccharide, the CXCL12γ chemokine binds substantially

less to its immobilized CXCR4 receptor. Interestingly, after the injection of 50nM CXCL12γ in the presence of HP12, the binding curve resembles more that of 50nM CXCL12α implying that when CXCL12γ is in the presence of HP12, thus with a 'blocked' C-terminal, it 'behaves' in a similar way to CXCL12α.

An alternative conclusion to this result may be that only a partial amount of CXCL12γ bound to the free HP12, inhibiting a percentage of the chemokine from binding the immobilized CXCR4 and that the small binding signal that is observed (Figure 5.7 B red curve) is that of the non-HP12-bound CXCL12γ binding the immobilized coreceptor. However, this conclusion is ruled out because a 5-fold molar excess of oligosaccharide (~278 nM) is used when incubated with the CXCL12γ chemokine (50 nM), thus there is virtually no chance of non-HP12-bound CXCL12γ binding the immobilized coreceptor.

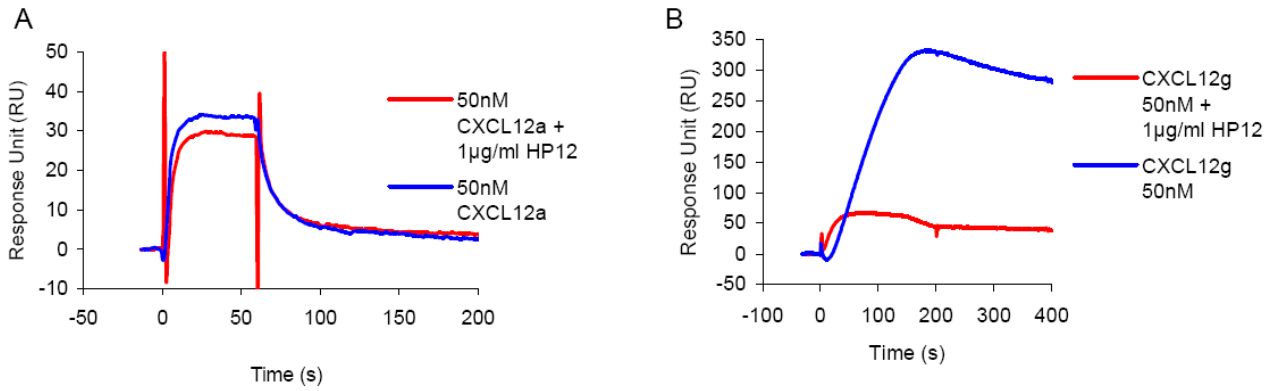


Figure 5.7 Effect of 1µg/ml HP12 (red) on binding of 50nM CXCL12α (blue) (A) and 50nM CXCL12γ (blue) (B) to CXCR4.

We confirmed this result in the context of mammalian Cf2Th cells where CXCR4 was in its natural membrane-bound environment. To investigate whether HP12, in the context of the cell surface, differently affected the binding of CXCL12α and CXCL12γ with cell surface CXCR4, we compared the absorption of these two isoforms onto the $CXCR4^{+}$ T lymphocyte cell line (CEM cells) by flow cytometry. Before this investigation, the absence of cell-surface GAGs required determination by flow cell cytometry so as to be sure of the absence of any competing / contaminating GAGs in the system. CEM cells express little to no GAGs on their cell surface, however, to make sure that all GAGs were absent, the cells were systematically treated with a cocktail of heparinase I and II and chondroitinase A, B, C prior to CXCL12 incubation (Figure 5.8). The following antibodies were used for the detection of GAGs: anit-chondroitin-6-sulphate, anti-chondroitin-4-sulphate and 4G10. Due to the fact that there is a very low expression of cell-surface GAGs on CEM cells, the digestion (using heparinase I and II and chondroitinase A, B, C) was also performed on the epithelial cells (Cf2Th cells, which display more pronounced GAG expression profile) to confirm the enzymes' functionality and efficacy (Figure 5.8).

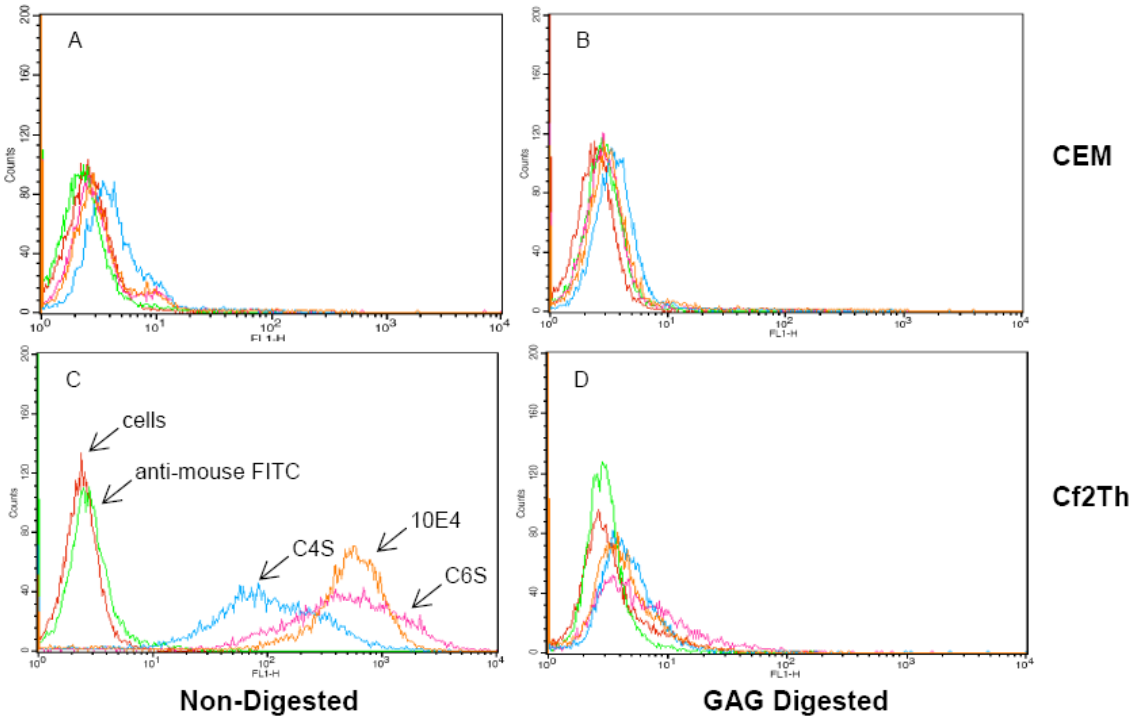


Figure 5.8 Systematic treatment of CEM cells with heparinase I and II and chondroitinase A, B,C prior to CXCL12 binding experiments. Cf2Th cell digestion is shown to illustrate the efficacy of the GAG digesting enzymes as there are little to no GAGs on the CEM cell surface. For all graphs, red = unstained cells only, green = anti-mouse FITC antibody, light blue = anti-chondroitin-4-sulphate, orange = anti-heparan sulphate (10E4) and pink = anti-chondroitin-4-sulphate. (A) Non-digested CEM cells, (B) GAG-digested CEM cells, (C) non-digested Cf2Th cells, (D) GAG-digested Cf2Th cells.

The GAG digest was thus effective as shown by the significant decrease in staining intensity of the antibodies (chondroitin-6-sulphate recognises, anti-chondroitin-4-sulphate and 4G10) in the Cf2Th cells. Since the pre-digest staining of the Cf2Th cells show a significant level of GAG expression and in the post-digestion essentially all cell-surface GAGs were removed, we can thus infer that the digestion was complete for the CEM cells too, despite the much lower level of initial GAG expression on these cells.

Now that all cell-associated GAGs were removed from the equation, we proceeded to test the binding of α-FITC labelled CXCL12α and IC12 labelled CXCL12γ binding to the GAG-digested CEM cells in the absence and presence of cell-free HP12 oligosaccarides.

The monoclonal antibody IC12 (from Fernando Arenzana-Seisdedos, Institut Pasteur) which recognises the C-terminal of CXCL12γ, was used for the experiments with CXCL12γ and a directly labelled CXCL12α-FITC was used for monitoring CXCL12α binding to cell-associated CXCR4 on GAG-digested cells. Data reported in Figure 5.9 A shows that the 1µg/ml HP12 had no effect on CXCL12α binding to CXCR4 (blue curve) as compared to CXCL12α binding CXCR4 in the absence of the oligosaccharide (pink curve). However in Figure 5.9 B, there is a significant displacement of the intensity to the left of the cell surface CXCR4-bound CXCL12γ in the presence of HP12 (blue curve) as compared to CXCL12γ alone (pink curve). The orange curve in Figure 5.9 B represents the background binding of IC12 to cell surface CXCR4. The green curves correspond to 12G5-FITC binding to verify for cell-surface CXCR4 expression. These results are in agreement with the biacore data showing that when HP oligosaccharides are pre-incubated with CXCL12γ and subsequently injected over or incubated with

CXCR4-expressing, GAG-digested cells, the association with CXCR4 is markedly decreased, which is not the case for CXCL12α. Thus, these data provide more evidence for the argument that the C-terminal of CXCL12γ, containing an elongated basic tail enriched in BXBB HS binding motifs, is also involved in binding to the CXCR4.

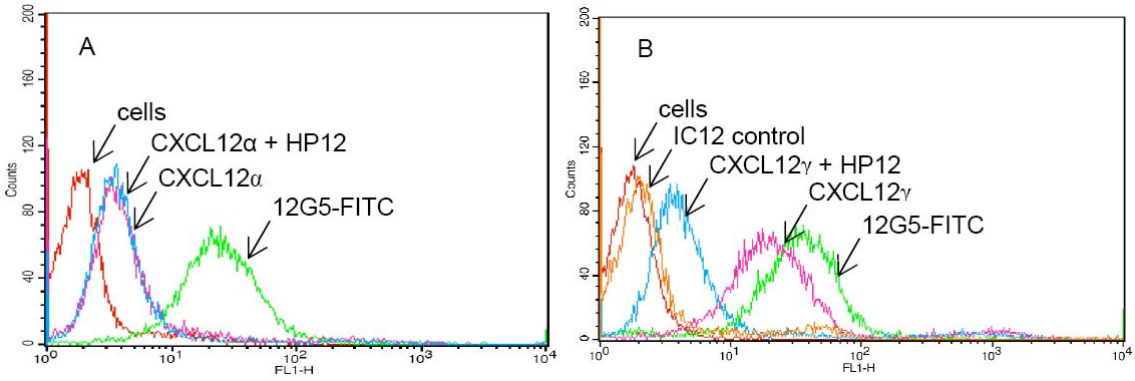
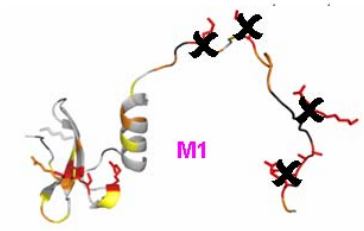


Figure 5.9 Effect of 1µg/ml HP12 on binding of 50nM CXCL12α and 50nM CXCL12γ to CXCR4 expressing, GAG-digested cells was tested using FACS analysis. CXCL12α (A) and CXCL12γ (B) binding to CXCR4 on CEM cells in the absence (magenta) and presence (blue) of 1µg/ml HP12. 12G5-FITC binding is shown (green) to demonstrate CXCR4 expression. The orange curve in (B) is the non-specific binding of the 1C12 antibody.

The mutant M1 is the CXCL12γ isoform where many (9) of the basic residues in the C-terminal tail were mutated into Serines in order to destroy the BBXB HS consensus binding sequences. Thus the following basic residues were mutated into Ser: Lys77, Lys78, Lys80, Lys83, Lys84, Arg86, Lys88, Lys89 and Lys91 (Figure 5.10).



M1 ** ****** KPVSLSYRCPCRFFESHVARANVKHLKILNTPNCALQIVARLKSNNRQVCIDPKLKWIQEYLDKALN KGRREEKVGSSESSIGSSKSSQSSRSAAQKRKN

Figure 5.10 The basic amino acids (K and R) that are highlighted in red in the sequences are amino acids that have been shown to be implicated in GAG-binding. ** KP signalling residues, **** RFFESH initial contact/docking site with receptor and GAG-binding domains (BBXB) are indicated in the CXCL12γ sequence by the black brackets and the structures of each isoform are shown. M1 is depicted, showing the BBXB HS binding motifs in the C-terminal being destroyed by mutation of certain basic residues to serines. Chemical shift variations upon GAG addition (dp4) are represented on M1 in colour; Red residues bind the most to GAGs and orange residues bind less and yellow residues bind the least (Laguri, Sadir et al. 2007).**

A further confirmation that the C-terminal of CXCL12γ was implicated in the high affinity binding of CXCL12γ with CXCR4 was the use of the mutant M1 in the SPR and FACS analysis. All the BXBB HS binding sites in the C-terminal of CXCL12γ have been removed by the mutation of 9 basic amino acids within these regions into Serines. As seen in Figure 5.11 A, 50nM M1 displays a much lower binding profile than that of CXCL12γ, and after the injection, M1 dissociates from the immobilized CXCR4 (binding curves eventually returned back to the baseline), as seen for CXCL12α. Also, as seen for the CXCL12α:CXCR4 interaction, HP12 had a minimal effect on the interaction between M1 and CXCR4. This was confirmed in the FACS analysis as pre-incubation of M1 with HP12 had no effect on the binding with the cell-surface CXCR4, as similarly seen with CXCL12α (Figure 5.11 B). Thus, the BXBB HS binding sites found in the C-terminal of CXCL12γ are responsible for the strong affinity between this chemokine and CXCR4 and HP12mer oligosaccharides are capable of disrupting this interaction.

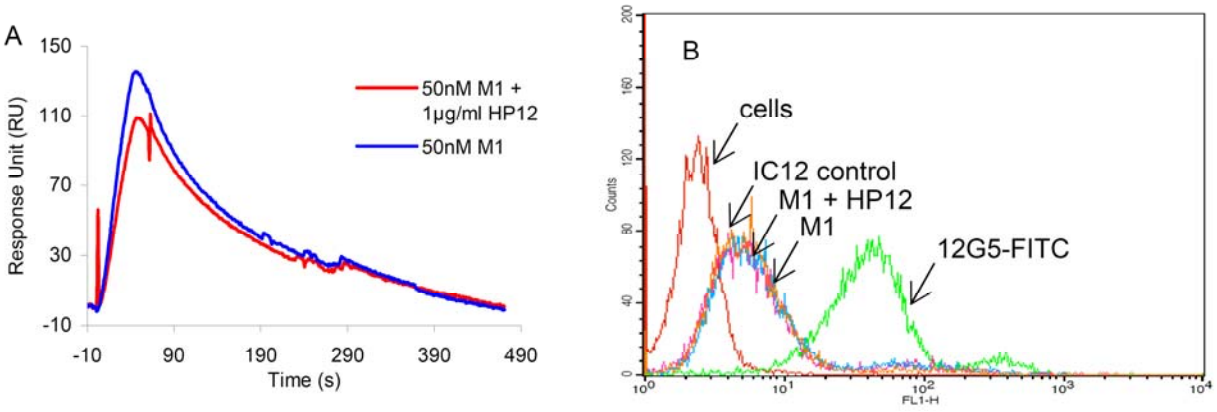


Figure 5.11 (A) Effect of 1µg/ml HP12 (red) on binding of 50nM M1 (blue) on immobilized CXCR4 and 50nM M1 in the presence (blue) and absence (magenta) of 1µg/ml HP12 binding to CXCR4 expressing CEM cells (red). 12G5-FITC (green) is shown to demonstrate CXCR4 expression (B).

5.3.5.1 Hypothesis for the Role played by GAGs in the context of signalling

Since CXCL12γ demonstrates a more significant interaction with CXCR4 due to its elongated and basic C-terminal and that this stable interaction is disrupted by HS12 oligosaccharides, we hypothesised that the N-terminal sulphotyrosines might be involved in the binding of CXCL12γ and that this may explain the differences observed between CXCL12γ and CXCL12α binding to CXCR4.

We decided to investigate this hypothesis using two experimental approaches; This phenomenon was investigated using Surface Plasmon Resonance (SPR) and Nuclear Magnetic Resonance (NMR) for the following reasons:

Initially, we wanted to verify the presence of the sulphotyrosines on the CXCR4 by the detection of a monoclonal antibody binding that specifically recognises sulphotyrosines, called anti-sulphotyrosine. The antibody bound to the CXCR4 surface, confirming the presence of the sulphotyrosines (Figure 5.12 A red curve, G). Following this, pre-incubation of the CXCR4 surface with 50nM CXCL12γ, blocked the mAb anti-sulphotyrosine from binding to the CXCR4 surface. As can

be seen in the blue curve of Figure 5.12 A and E, CXCL12γ is injected onto the CXCR4 surface from -500 to 0 RU on the y-axis showing a strong binding response, after which 0.5µg/ml antisulphotyrosine is injected and appears not to bind at all to the surface. In order to rule out the concern that the bulky monoclonal anti-sulphotyrosine antibody was not sterically restricted from binding to the sulphotyrosines once CXCL12γ was already bound, we performed the same experiment but with an antibody (4G10) that binds the first 38 N-terminal amino acids of CXCR4, independently of the presence of the sulphate groups (Figure 5.12 B red curve). Here, it was shown that once CXCL12γ was bound to the CXCR4 surface, an injection of 0.5µg/ml 4G10 is also able to recognise and bind the N-terminal of CXCR4. This proves that there is no steric hindrance between CXCL12γ binding and anti-sulphotyrosine, and that the binding site of anti-sulphotyrosine and that of CXCL12γ are mutually exclusive unlike 4G10 and CXCL12γ.

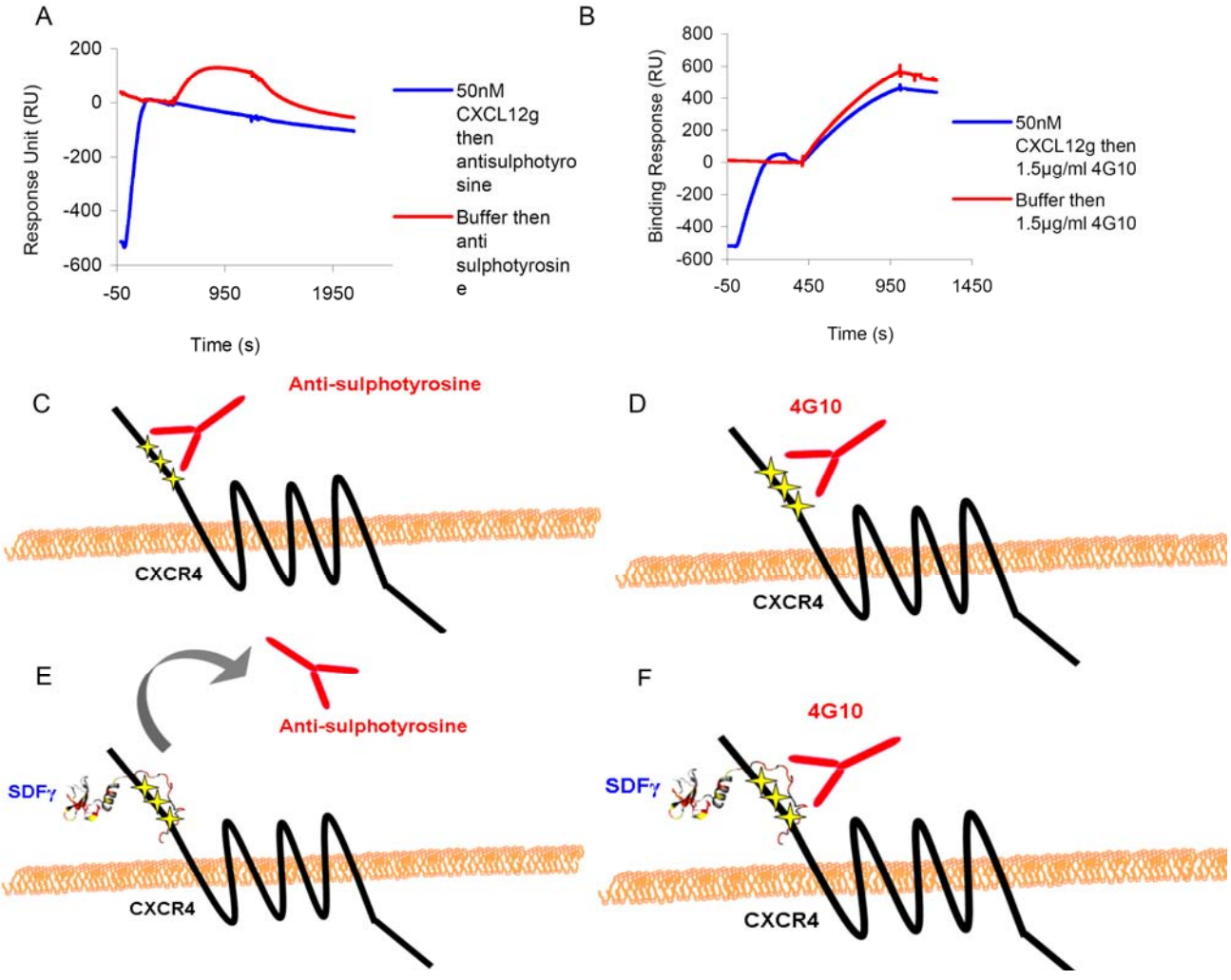


Figure 5.12 Injection of antisulphotyrosine alone over the CXCR4 surface (A [red curve], C) and injection of 50nM CXCL12γ onto CXCR4 directly followed by antisulphotyrosine (A [blue curve], E). The control injection of 4G10 onto the CXCR4 surface alone (B [red curve], D) or injection of 50nM CXCL12γ onto CXCR4 directly followed by 4G10 (B [blue curve], F).

This result has been further investigated using the second technique, NMR spectroscopy. NMR is a complex type of spectroscopy that allows one to see where each residue in the protein places in space and in relation to the surrounding residues as it can provide information on every atom that has an odd number of protons e.g.^{1}H, ^{13}C and ^{15}N. Very simply put, proteins are hit with radio waves while they're in a strong homogeneous magnetic field which causes the protons of each atom in the protein to align (nuclear magnetic dipoles). The radio wave causes the nuclear dipoles to begin to tilt and when the radio wave is stopped, the nuclear dipoles return to their original orientation. Since each amide ^{1}H and ^{15}N are within a distinct environment (in terms of their neighbouring residues in the peptide sequence and neighbouring residues in the three-dimensional structure) the resonance frequencies of each amide ^{1}H and ^{15}N will differ slightly from one another. It is these slight differences in resonance frequencies that are plotted on a two dimensional grid reflecting the chemical shift (change in resonance frequency) of each amide ^{15}N in the protein as a function of its corresponding amide ^{1}H shift (Figure 5.13 B). The ^{15}N-^{1}H amide chemical shifts for CXCL12γ were monitored in the presence of either a sulphated or non-sulphated N-Terminal CXCR4 peptide. Thus, each residue in the protein is depicted as a dot on the Heteronuclear Multiple Quantum Correlation (HMQC) graph and consequently when the protein undergoes a structural re-arrangement due to the presence of a ligand (in this case, the N-terminal of CXCR4), the chemical shift between the two states (bound and un-bound) can be compared for *each* residue. Hence we can detect precisely which residues moved (and by how much) when the different ligands were added. Therefore, providing that each residue is well depicted in the HMQC plot, NMR is a very appropriate technique for determining the precise residues that are involved in a specific binding interaction.

Here, recombinant ^{15}N labelled CXCL12γ was titrated with either a sulphated or non-sulphated chemically synthesized CXCR4 N-terminal peptide. The peptides comprised the first 29 amino acids of the CXCR4 N-Terminus: MEGISIYTSDNYTEEMGSGDYDSMKEPAF. Residue C28 was replaced with an alanine so as to prevent oxidative peptide dimer formation (Veldkamp, Seibert et al. 2006). The interaction was monitored for two different peptides, one containing 3 sulphotyrosines at positions 7, 12 and 21 and the other peptide contained un-modified tyrosines (peptide synthesis by collaboration with Françoise Baleux, Institut Pasteur). Briefly, 100μM ^{15}N labelled CXCL12γ was inserted into an NMR tube and either the non-sulphated or sulphated N-terminal peptides were titrated into the CXCL12γ in the NMR buffer (20mM Na-Phosphate pH 5.7, 0.01% azide, 2% complete protease inhibitors, 10% $^{2}H_2O$). Spectra were recorded after each incremental addition of the peptide (non-sulphated or sulphated) and little/no precipitation was observed when the peptide was added.

When the recombinant ^{15}N labelled CXCL12γ was titrated with the non-sulphated CXCR4 N-terminal peptide, the core domain (the part of CXCL12γ that corresponds to the CXCL12α isoform, first 68 amino acids [CXCL12$\gamma_{1\text{-}68}$]) displayed chemical shifts, and nothing was seen in the C-terminal of CXCL12γ. Interestingly, a similar result was found by Veldkamp and colleagues where CXCL12α was titrated with non-sulphated N-Terminal CXCR4 peptide, the

region 1-68 of CXCL12α was bound. We found exactly same chemical shifts for the CXCL12$\gamma_{1\text{-}68}$ bound to the non-sulphated peptide (the red bars in the graph in Figure 5.13 A), independently of the C-terminal of CXCL12γ. Veldkamp and colleagues also demonstrated that when CXCL12α was titrated with a single-sulphated (sulphated on Tyr 21) N-Terminal CXCR4 peptide, no changes were seen on the CXCL12α (Veldkamp, Seibert et al. 2006). This means that the sulphates on the N-terminal CXCR4 peptide are not significantly involved in the contact with the 1-68 domain of CXCL12α.

When the recombinant ^{15}N labelled CXCL12γ was titrated with the sulphated N-terminal CXCR4 peptide, the CXCL12$\gamma_{1\text{-}68}$ part of CXCL12γ interacted with the sulphated peptide in exactly same manner as it did with the non-sulphated N-Terminal CXCR4 peptide – thus confirming the results of Veldkamp and colleagues that the sulphates are not important to the interaction with the 1-68 domain. It was re-assuring that our results for CXCL12$\gamma_{1\text{-}68}$ mirrored those that Veldkamp and colleagues obtained for CXCL12α and it proves that our protein is functional and that the experiment was performed correctly. However, when the labelled CXCL12γ came into contact with the sulphated N-Terminal CXCR4 peptide, the C-Terminal of CXCL12γ was also modified (Figure 5.13 A blue bars). Therefore, the presence of the sulphates on the N-Terminal of CXCR4, enables the CXCR4 peptide to bind the C-Terminal of CXCL12γ.

The following individual residues were found to be implicated in the interaction surface between CXCL12$\gamma_{1\text{-}68}$ and the sulphated N-terminal peptide involving the N-loop: (F13), β1(K24,H25), β2(39-42), β3(48-50) and the α-helix (W57, Y61, L62). The CXCL12γ core domain (CXCL12$\gamma_{1\text{-}68}$) strongly binds the N-terminal of CXCR4 (with and without the sulphated tyrosines) – the residues that are involved in the binding of the N-terminal are denoted by the red bars and the height of the bar corresponds to the intensity of the interaction (Figure 5.13 A). Exactly the same chemical shifts were observed for the CXCL12α in Veldkamp and colleagues' work.

The sulphated CXCR4 peptide causes stronger chemical shifts on the CXCL12γ isoform compared with the non-sulphated peptide, particularly in the C-terminal basic extension compared to the non-sulphated peptide. These chemical shifts are quite low, thus they do not represent a large movement of the residues in the presence of the sulphated peptide. However, due to the highly disordered and flexible nature of the CXCL12γ C-terminal and the fact that there are multiple BBXB motifs all possessing similar amino acid repeats, all the specific residues that participate in the liaison with the sulphated N-terminal CXCR4 peptide are not able to be pin-pointed. Thus, we are not able to detect the chemical shift data for several amino acid residues in the C-terminal of CXCL12γ, which may in fact be high – but are undetectable with this technique. Despite this, a large group of amide peaks (residues 68-98) in the chemokine C-terminal displayed significant chemical shifts in the presence of the sulphated peptide (Figure 5.10 B CXCL12γ C-Ter). This suggests that sulphotyrosines in the CXCR4 N-terminal strengthen the interaction with CXCL12γ, and that the C-terminus of the chemokine is responsible for the increased affinity with the coreceptor.

NMR experiments show that the interaction of CXCL12γ with the CXCR4 peptides involves both the N-terminal folded domain of the chemokine, as well as the C-terminal tail.

Unlike CXCL12α as shown in Veldkamp *et al.*, (Veldkamp, Seibert et al. 2006) in our experiment, CXCL12γ does not seem to dimerise. It remains to be confirmed, however, we believe that CXCL12γ does not dimerise in the presence of the N-terminal of CXCR4. The functional ramifications for this phenomenon are yet to be elucidated.

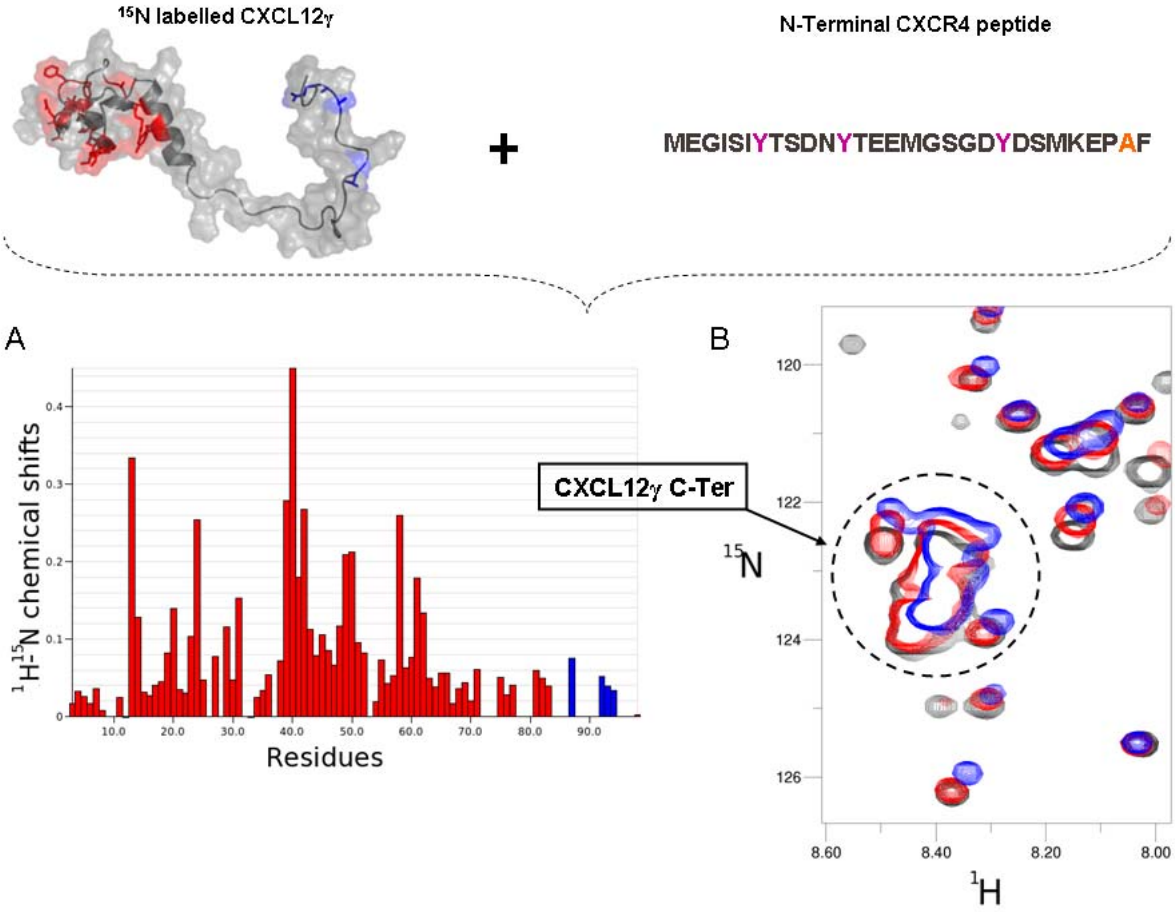


Figure 5.13 ^{15}N labelled CXCL12γ was observed interacting with chemically synthesized peptides comprising the first 29 amino acids of the CXCR4 N-Terminus. (A) Amino acid residues that interact with both the sulphated and non-sulphated peptide (shown in red) and those that interact with the sulphated peptide (shown in blue). The amino acids that interact exclusively with the sulphated peptide are found both in the core domain and in the C-terminal of CXCL12γ. (B) The chemical shift perturbation observed for the CXCL12γ interacting with the non-sulphated peptide (red) overlayed with the chemical shift pertubations observed for CXCL12γ interacting with the sulphated peptide (blue) in the C-terminal region (CXCL12γ C-Ter). Single amino acids cannot be determined due to the repeating BBXB motifs in the CXCL12γ C-terminal and its non-structured mobility.

To further confirm the data using a different experiment, we immobilized either the sulphated or non-sulphated peptides through amine coupling chemistry onto 96 well plates and tried to determine the binding of CXCL12γ or the C-terminal of CXCL12γ. However, these experiments still require optimization to be demonstrative.

Based on the above data and from previously proposed ideas of the role played by GAGs *in vivo* (Kuschert, Coulin et al. 1999), we propose a model for the role of glycosaminoglycans and CXCR4 in modulating chemokine activity, summarised here as a "chemokine interactome" (Figure 5.14) .Five scenarios are depicted for CXCL12 signalling through CXCR4 and the role played by GAGs is depicted in the cartoon. CXCL12α signals through CXCR4 via the interaction of its N-terminal residues within the transmembrane region of CXCR4 (1), however, at equivalent concentrations, CXCL12γ is known to be inhibited from signalling through CXCR4, and we propose that this could be due to the liaison of the basic C-terminal of CXCL12γ with the anionic N-terminal of CXCR4 which posseses sulphotyrosines (yellow stars). This interaction would place the N-terminal of CXCL12γ too far from the site of activation within CXCR4 (2). When in the presence of GAGs (3), CXCL12α signalling is possible through the CXCR4 coreceptor as the GAG binding site and the CXCR4 binding site do not overlap (Crump, Gong et al. 1997; Amara, Lorthioir et al. 1999; Sadir, Baleux et al. 2001). In the case of CXCL12γ, the presence of GAGs could increase the signalling capacity of CXCL12γ as the anionic GAG oligosaccharide competitively binds the basic C-terminal of CXCL12γ, displacing it from the N-terminal of CXCR4. In doing so it would sterically liberate CXCL12γ and allow the N-terminal of the chemokine to easily access its activation site within the transmembrane region of CXCR4 (4).

In terms of the oligomerisation of chemokines, the dimer form of CXCL12α has been shown to bind GAGs and when bound to CXCR4, induces Ca^{2+} mobilization but inhibits chemotaxis (Veldkamp, Seibert et al. 2008). Based on the recent crystal structure of the homodimer CXCR4 (Wu, Chien et al. 2010), we propose that the CXCL12α dimer, when bound to a homodimer of CXCR4, interacts with two N-termini of the two CXCR4 molecules, thus restricting the movement of the N-terminal of the CXCL12α dimer within the extracellular loops of CXCR4 and thus does not induce cell migration.

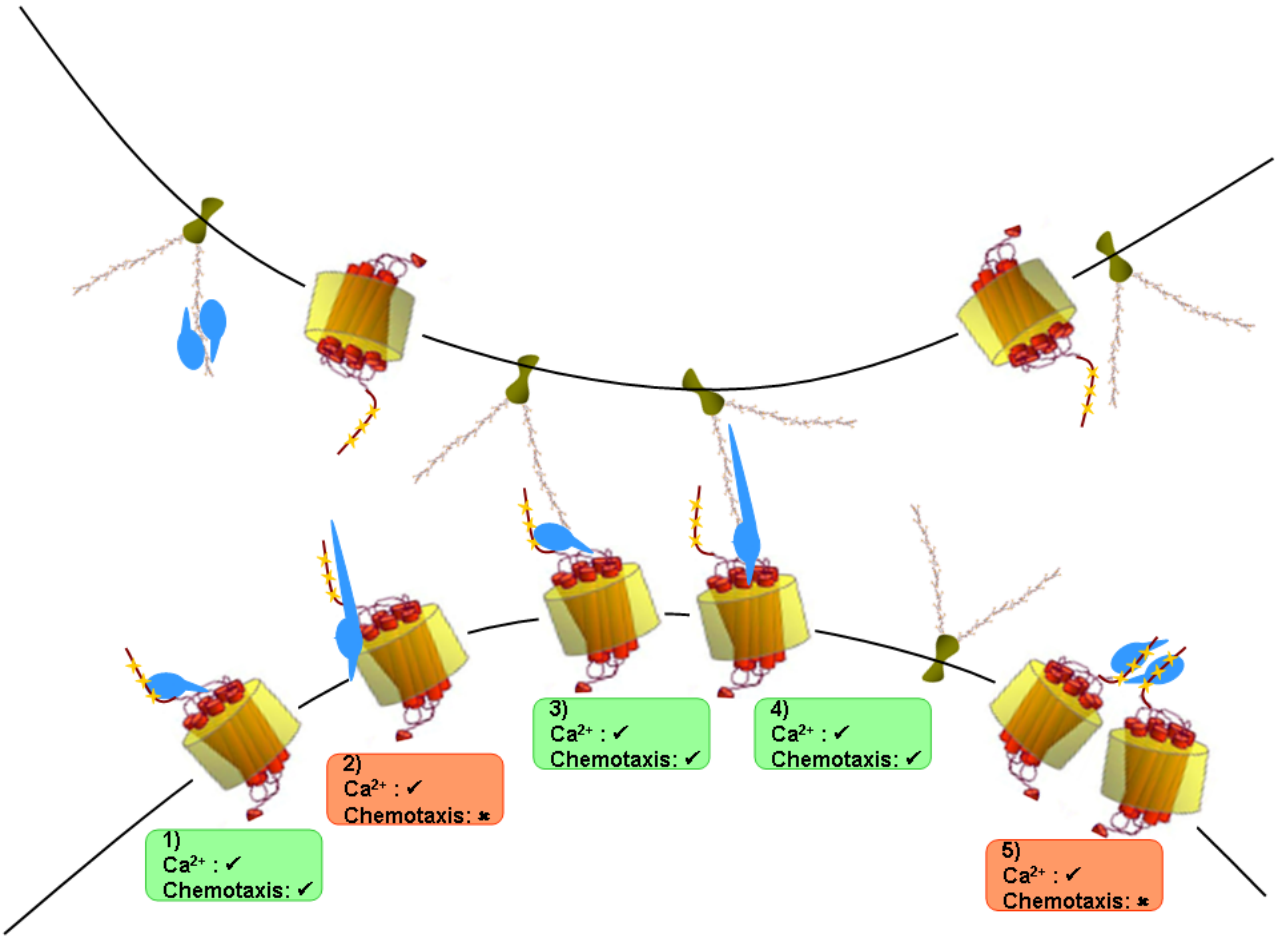


Figure 5.14 Proposed "chemokine interactome". CXCL12α monomers signal similarly through CXCR4 in the absence (1) and presence (3) of GAGs. However, CXCL12γ signals very weakly though CXCR4 in the absence of GAGs (2) and a stronger signalling is seen in the presence of GAGs [preliminary data] (4). Cell-surface glycosaminoglycans can induce dimer formation of the CXCL12α chemokine as can the sulphated N-terminal of CXCR4, which does not result in functional chemotaxis (5).

5.4 Discussion

Analysing the binding interactions between GPCRs (CCR5 and CXCR4) and their ligands in real-time and without the need for labeling is hugely advantageous. Not only are these receptors the coreceptors for HIV-1 (and assessing the ability of molecules to target the coreceptor binding site of gp120 would be possible with such a platform), but very little is known about how CXCL12 and the different isoforms bind CXCR4 and about the role played by heparan sulphate.

We chose to use the surface plasmon binding assay as this technique has many advantages; it allows for the isolation of purified coreceptors from their natural environment and their immobilization for subsequent interaction analysis. The solubilized coreceptors are not labeled (although tagged) and neither are their ligands which allows the study of an isolated interaction where there is slight possibility for non-specific binding. SPR allows for the generation of real-time binding data which makes it a technique that is well suited to the analysis of binding kinetics. The association and dissociation rate constants and equilibrium constants are all easily calculated when a range of concentrations of the analyte are injected over the ligand, regenerating the surface in between injections.

However, this technique does come with certain difficulties too: this assay took almost two years of relentless optimisation of the solubilization buffer, chip surface type and running buffer conditions.

Other techniques that are used to measure the equilibrium dissociation constants (K_D) are often performed with labelled ligands (Rueda, Balabanian et al. 2008) which might change the three-dimentional conformation of the protein and therefore change its binding properties and affinity for its receptor. In the latter case, coreceptors are often used in their cell-bound environment for affinity tests and thus cell-associated glycosaminoglycans and other cell bound receptors are often present on the cell surface. Such molecules include sphingolipids, like HS, which can be bound by CXCL12 (Sandhoff, Grieshaber et al. 2005). These other molecules, in particular GAGs, can bind to the ligands and contribute to a false positive signal of ligands binding to the receptors on the cell surface. This would be particularly true for CXCL12γ which binds to a range of different GAGs. In other studies, proteoliposomes or pseudovirions containing CXCR4 are captured onto the biacore surface and kinetic interaction studies are performed with ligands that are injected onto the proteoliposome / pseudovirion surface. This approach can also cause non-specific binding between the ligand and the large structures of the proteoliposomes and pseudovirions (Hoffman, Canziani et al. 2000; Zhukovsky, Basmaciogullari et al. 2010). For these reasons, native solubilization, purification and stabilization of the receptors outside of the cell / virion / proteoliposome, is crucial to understanding their function.

Although manipulating GPCRs is a challenging task based on their transmembrane nature, detergent/lipid containing cocktails have been used to solubilize these proteins from their native membranes (Navratilova, Sodroski et al. 2005). We slightly modified this solubilization cocktail and obtained reproducible binding data for the two CXCL12 isoforms and antibodies binding to the solubilized immobilized coreceptors.

Here we report a K_D of 13 ± 1.6 nM for CXCL12α and CXCR4 comparable to those obtained with either a similar technique or cellular systems where the coreceptors remained in their natural environment (Di Salvo, Koch et al. 2000; Navratilova, Dioszegi et al. 2006). Also for the first time, the affinity was estimated for CXCL12γ and CXCR4 which is $K_D = 0.7 \pm 0.3$ nM. Fitting of the curves was complicated due to the complex buffer used and the mass transport effects created by the very high on rates (that exceed that of diffusion) for the CXCL12γ-CXCR4 binding data. Thus, the calculated affinities reported here should be considered as estimates.

Using both surface plasmon resonance and flow cell cytometry we have shown that CXCL12α binds to CXCR4 and that this interaction is not influenced by the presence of glycosaminoglycans, however, the CXCL12γ isoform which has the non-structured basic C-terminal, displays a radical reduction in binding to the coreceptor in the presence of HP. We hypothesize that this reduced binding is due to the reduced binding of the basic residues in the chemokine C-terminal with the sulphotyrosines in the N-terminal of CXCR4 due to the competition with the sulphated oligosaccharides. This is supported by the fact that once CXCL12γ is bound to immobilized CXCR4, mAb anti-sulphotyrosine can no longer recognise

the sulphotyrosines in the N-terminal of CXCR4 as they are presumably bound and 'hidden' by the chemokine binding the N-terminal of CXCR4.

What are the implications of this apparent increased binding of CXCL12γ to CXCR4 in the absence of GAGs and the apparent decreased binding in the presence of GAGs? Could CXCL12γ be a kind of antagonist since its binds strongly to CXCR4 although it signals poorly? Or CXCL12γ could be a chemokine that is secreted for low and prolonged/sustained levels of signalling due to its high affinity for the cell-surface GAGs and CXCR4. CXCL12γ contains several serine-protease cleavage sites in its C-terminal region and it has been speculated that (as for VEGF-A, vascular endothelial growth factor A), HS interacting with CXCL12γ protects this domain from proteolytic attack, therefore contributing to the prolonged immobilization and increased half-life of CXCL12γ in tissues (Rueda, Balabanian et al. 2008). Following from this hypothesis, the proteolytic attack and removal of the C-terminal from CXCL12γ, can also be a mechanism for regulating CXCL12γ signalling effects, through the release of the CXCL12α-like domain. Cell-based signalling assays need to be performed to verify the exact role and mechanism of action of the CXCL12γ and the role played by GAGs *in vivo* in healthy organisms and those in disease states.

From a spatial stoichiometric point of view, when CXCL12γ is bound to the N-terminal of CXCR4, it might not reach the activation domain (embedded between the transmembrane helices) of CXCR4 easily if the C-terminal of the molecule is bound tightly to the N-terminal of CXCR4. Thus in the presence of HP, the electrostatic forces cause the binding of the oligosaccharide to the basic C-Terminal and the CXCL12γ is 'detached' from the N-terminal of CXCR4 and can reorient itself to trigger its receptor. In addition, NMR analysis showed that the sulphations in the N-terminal of CXCR4 cause chemical shifts in the residues within the C-Terminal of CXCL12γ, and therefore the C-terminus of the chemokine is involved in the binding of the sulphations in the N-terminal of CXCR4, based on the stronger chemical shifts observed for the chemokine in presence of the sulphated peptide as compared to the non-sulphated peptide.

The first 68 residues of CXCL12γ adopt a structure that is closely related to CXCL12α and attached to this is a highly unstructured and flexible 30 amino acid C-Terminal as was shown by NMR (Laguri, Sadir et al. 2007). Proteins with such disordered regions are believed to perform critical functions, including molecular recognition through large and accessible interaction surfaces. Thus, due to the highly basic nature and disordered state of the CXCL12γ C-terminal as well as the importance of glycosaminoglycan (GAG) recognition for chemokine function, it is no surprise that CXCL12γ binds a range of GAGs (Laguri, Sadir et al. 2007). Heparan sulphate (HS) oligosaccharides are ubiquitously found on the cell surface and within the extracellular matrix (Bernfield, Gotte et al. 1999). These highly sulphated molecules are implicated in protein regulation and they play a major role in chemokine immobilization and the formation of haptotactic gradients of chemokines along the cell surfaces thus providing directional cues for migrating cells (Campanella, Grimm et al. 2006).

The chemokine residues that are involved in HS binding are well defined for CXCL12α, which include K24, K27 and R41 (Sadir, Baleux et al. 2001). NMR

analysis of a ^{15}N-^{13}C-CXCL12γ/dp4 HP complex revealed two binding domains, one on the core, which includes K24 and R41 but also includes (R20, V23, K24, A40, R41 and N45) and another within the domain of the C-terminal extension (K83 – K97) which stabilizes the complex with the GAGs (Laguri, Sadir et al. 2007).

A number of other proteins that are involved in different systems also posses elongated, basic C-Terminal domains that play an important role in the proteins' function; CCL21 is a chemokine which signals through the CCR7 GPCR required for the mobilization of dendritic cells to the lymphoid tissues. CCL21 possesses a highly basic C-terminal (40 amino acids of which 12 are Lys or Arg) tail which has been suggested to interact with GAGs (as does that of CXCL12γ) to in order to facilitate the formation and maintenance of CCL21 gradients (Hirose, Kawashima et al. 2002). CCL21 causes random dendritic cell movement as it triggers integrin-mediated adhesion when it is bound to the surface through its GAG-binding C-Terminal domain. Dendritic cell specific proteases are able to cleave the C-Terminus of CCL21, in order to release a soluble fragment that can diffuse, form gradients and provide a second nested chemotactic signal, thus resembling the soluble CCR7 ligand, CCL19. Schumann and colleagues propose that HS-bound-CCL21 triggers adhesion, random polarisation and migration of dendritic cells, whereas soluble chemokine gradients introduce directional bias. Thus CCL21 has both an adhesive and a chemotactic function implying that the mode of chemokine presentation may determine the cellular response (Schumann, Lammermann et al. 2010); this may also be the case for CXCL12α and CXCL12γ.

C-terminal extensions enriched in basic residues binding to DNA have been documented in the context of DNA-binding proteins. Such extended basic tails increase the affinity for DNA and can selectively either activate or repress gene transcription (Crane-Robinson, Dragan et al. 2006). Also, extended basic C-terminals have been documented in helicase proteins which bind to and tether RNA for subsequent unwinding (Mallam, Jarmoskaite et al. 2011). Thus, it is not un-common for proteins to have unstructured basic C-terminal tails that serve to tether anionic binding partners. However, this is the first ever documented instance of a GAG oligosaccharide that regulates the binding of such basic C-terminal tails in chemokines to their binding partners.

Tyrosine sulphation is a post-translational modification of certain secreted and membrane-bound proteins, however its biological role and regulation have been unclear. Recent studies have implicated tyrosine sulphation as a determinant of protein-protein interactions involved in leukocyte adhesion, haemostasis and chemokine signalling (Kehoe and Bertozzi 2000). Farzan *et al.,* showed in 1999 that the HIV-1 chemokine coreceptors, CCR5 and CXCR4 are tyrosine sulphated (Farzan, Mirzabekov et al. 1999) and that removal of the CCR5 sulphates, either with sodium chlorate treatment or by mutation of the sulphotyrosine residues, decreased natural chemokine ligand binding and HIV-1 infection respectively (Farzan, Mirzabekov et al. 1999). The importance of this sulphation to the function of CXCR4 suggests that regulation of this modification could be useful in the modulation of immune function or in disease states in which chemokine receptors participate (Farzan, Mirzabekov et al. 1999; Bannert, Craig et al. 2001).

The sulphotyrosines found in the N-terminus of CCR5 are known to bind a surface at the intersection between the bridging sheet and the V3 loop in HIV-1 gp120 to allow entry. A good electrostatic complementarity was observed between the acidic N-terminal of CCR5 and the basic bridging sheet of the gp120 seen from NMR and crystallographic structures (Huang, Lam et al. 2007). More recently, Veldkamp *et al.,* demonstrated that the sulphotyrosines occurring in the N-terminal of CXCR4 interact with precise basic residues within CXCL12α (Veldkamp, Seibert et al. 2006; Huang, Lam et al. 2007; Veldkamp, Seibert et al. 2008). Since we have established experimentally that the basic C-terminal of CXCL12γ binds both anionic heparan sulphate as well as CXCR4 – we thus hypothesised that the three sulphotyrosines present on the N-terminal of CXCR4 could contribute to CXCL12γ recognition through the basic C-terminal region.

There is a lot still to be elucidated about the signalling specificity and regulation of function of the CXCL12γ isoform. However, what is known is that the unusual, unstructured basic C-terminal of CXCL12γ displays an enormous structural plasticity and thus enables the protein to bind to multiple partners (GAGs or anionic residues on proteins). Another interesting feature of CXCL12γ is its decreased motility due to its elongated basic C-terminal. Once CXCL12γ is expressed and secreted from its parent cell, it will move out of its original tissue compartment at a much slower rate and will access the circulation with much slower kinetics (as compared to the CXCL12α isoform) due to its higher affinity for GAGs. One could speculate that CXCL12γ signalling is much more localized than that of CXCL12α due to this slower diffusion within tissue. Also, depending on the composition and extent of GAG sulphation levels (depending on the cell type, developmental stage and pathophysiological state of the cell (Turnbull, Powell et al. 2001)), the CXCL12γ might display different degrees of kinetics in relation to the extent of sulphation. This however, all still needs to be investigated.

In the presence of GAGs, CXCL12α dimerises (Veldkamp, Peterson et al. 2005) and here the GAG-induced dimer can bind the CXCR4 homodimer, as suggested by Wu *et al.,* in their model and by Veldkamp *et al.,* from their NMR structure of CXCL12α in complex with the N-terminal of CXCR4 (Veldkamp, Seibert et al. 2008; Wu, Chien et al. 2010). There is no current data as to whether a CXCL12α dimer can bind a homodimer of CXCR4 or a heterodimer of CXCR4 and CXCR7.

In the absence of GAGs, as has been hypothesized from Wu *et al.,* the crystal structure of the CXCR4 homodimer, a monomer of CXCL12α can bind to a homodimer of CXCR4. The CXCL12α could bind and activate the same CXCR4 or the CXCL12α could bind one CXCR4 monomer of the homodimer with its core region and then activate the neighbouring monomer of the homodimer with its first two N-terminal residues, *in cis*. One hypothesis is that the role played by the N-terminal of CXCR4 is to displace the GAGs that have bound to the CXCL12α chemokine dimer and allow for signalisation through the receptor (with a 1:1 binding of N-terminal and chemokine). However, it has been proposed that a 2:2 complex of N-terminal and chemokine is a partial agonist and selective antagonist as it can stimulate Ca^{2+} mobilization but cannot stimulate chemotaxis and is thus non-functional. This can be seen as a level of regulation of chemokine signalling (Veldkamp, Seibert et al. 2008).

Heterodimers including CXCR4 can form too. Levoye *et al.,* show that CXCR7 heterodimerises with CXCR4 and that CXCR7 expression impairs CXCR4-prompted $G_{\alpha i}$ protein activation and calcium responses (Levoye, Balabanian et al. 2009). CXCR7 is phylogenetically homologous to GPCRs and it fails to activate the inactive linked $G_{\alpha i}$ proteins, however it does induce CXCL12α receptor mediated cellular responses (Balabanian, Lagane et al. 2005; Naumann, Cameroni et al. 2010). The binding of CXCL12α to CXCR7 is very controversial, as this interaction was recently discoved and there are a lot of unknowns about this binding interaction and its role in CXCL12α signalling regulation. Naumann and colleagues described the occurrence of CXCL12α binding to CXCR7 and that CXCR7 acts as a scavenger for CXCL12α; CXCR7 internalises CXCL12α and targets it for degradation (Naumann, Cameroni et al. 2010). Thus, a possible scenario of binding could be a monomer of CXCL12α binding to a heterodimer of CXCR4 and CXCR7, causing the internalisation and degradation of CXCL12α in order to regulate the chemotactic activity of CXCR4. The issue of CXCL12α binding a CXCR4-CXCR7 heterodimer is very interesting in terms of its functional role for chemokine-mediated signalling and this needs to be further investigated.

We propose a hypothesis whereby a heterodimer of CXCR4 and CXCR7 can exist, or a homodimer of CXCR4 exists and CXCL12γ is bound to the N-Terminal of one of the CXCR4's in either the heterodimer or the homodimer. Once the CXCL12γ chemokine is 'tethered' or 'captured' to one of the CXCR4's, its elongated flexible C-Terminal allows it the mobility to bind and activate the adjacent coreceptor, being either the CXCR7 or the CXCR4. CXCR7 possesses only one tyrosine in its N-Terminal domain, and the literature thus far does not mention that this tyrosine is sulphated. Thus CXCL12α and CXCL12γ could bind to CXCR7 in an identical way, which is not the case as for CXCR4 (Rueda, Balabanian et al. 2008). Therefore, CXCL12γ may activate CXCR7 in a similar way to that of CXCL12α. Further experimentation is required to elucidate this hypothesis.

For the first time, we show that sulphated oligosaccharides differently influence the binding of CXCL12α and CXCL12γ to their cognate receptor, CXCR4 and that this modulation may play a fundamental role in the regulation of cell signalling, leukocyte trafficking and activation and cell migration.

The binding difference between CXCL12α and CXCL12γ for their receptor, CXCR4, is due to two important factors: i) the basic unstructured C-terminal of CXCL12γ that is not present in the CXCL12α isoform allows CXCL12γ to have a higher affinity for the ii) anionic N-Terminal of CXCR4 which contains sulphotyrosines. This has been shown by surface plasmon resonance and NMR.

Chapter 6: A synthetic heparan sulfate-mimetic peptide conjugated to a mini CD4 displays very high anti-HIV-1 activity independently of coreceptor usage

6.1 Un mimétique synthétique de l'héparan sulfate-conjugué à un peptide mini CD4 a une activité anti-VIH-1 très élevée indépendamment de l'utilisation des corécepteurs (sommaire en français)

Une thérapie anti-rétrovirale même très active (ARV) ne peut pas éradiquer complètement le virus VIH-1, ce qui explique pourquoi de nouvelles stratégies thérapeutiques, tels que les inhibiteurs de l'entrée virale sont nécessaires. L'entrée du VIH-1 est un processus complexe, qui offre de multiples sites à cibler pour l'intervention thérapeutique. Parmi eux, la surface de liaison du corécepteur - gp120, qui est hautement conservée dans de nombreuses souches VIH-1, est particulièrement attrayante. Cette région devient toutefois exposée, et donc sensible à l'inhibition, seulement de façon transitoire et dans un espace restreint stériquement, lorsque le virus a déjà été lié par la surface des cellules CD4 et que le processus d'entrée est largement engagé. Une molécule hybride (mCD4-HS_{12}) constituée d'un peptide mimétique de CD4 couplé à un dodécasaccharide héparane sulfate synthétique (HS_{12}) est capable d'empêcher à la fois l'attachement et l'entrée du VIH-1 R5 et X4 avec un IC_{50} 1-5 nM dans un test en culture cellulaire (Baleux, Loureiro-Morais et al. 2009). Cette activité est due à la formation induite du domaine de liaison sur le corécepteur via le groupement mCD4 de la molécule, suivie par l'interaction de forte affinité de l'HS_{12} anionique avec le corécepteur. Les HS sont extrêmement complexes dans la nature et leur complexité découle du nombre et de la position des groupes sulfates le long de la chaîne. Ainsi les sites exacts de contact entre la molécule sulfatée d'HS et la gp120 nécessitent d'être étudiés pour pouvoir créer une interaction plus spécifique et de plus forte affinité. Afin de réaliser ceci, nous avons d'abord développé et validé une plateforme utilisant la technologie des biocapteurs SPR dans laquelle des ligands de gp120, y compris les CD4, HS, les anticorps et corécepteurs solubilisés (CCR5 et CXCR4) sont immobilisés à la surface de la sensorchip, (dans un environnement lipide / détergent pour les co-récepteurs). Nous pouvons mesurer directement en solution et en temps réel les interactions de liaison entre la gp120 ou des complexes gp120-CD4 avec les récepteurs mentionnés ci-dessus dans un environnement sans étiquette. Ensuite, la plate-forme a été utilisée pour le criblage de banques de sucres basées sur des molécules HS différemment sulfatée et autres composés naturels mimant les HS. Compte tenu de ces approches, nous avons également conçu une série de tridécapeptides «$S(XDXS)_3$ » imitant la dodécamère héparane sulfate qui a déjà été montré pour cibler le site de liaison de la gp120 au corécepteur. Nous avons montré que l'un de ces composés (où X est une sulfotyrosine), lorsqu'il est lié de façon covalente à un mini-CD4 (mCD4-$P3YSO_3$) inhibe la liaison des gp120 qu'il soit R5 ou X4-tropique, aux molécules

suivantes : CD4, des anticorps dirigés contre le domaine CD4-induit, et CCR5 ou CXCR4. L'analyse de liaison est en faveur d'un mécanisme bivalent où le fragment mCD4 se lie d'abord, provoquant l'ouverture du site pour le corécepteur puis un fort blocage ultérieur par le tridécapeptide. Le conjugué a été beaucoup plus efficace qu'un mélange de mCD4 et tridécapeptide isolés, ce qui indique que la liaison covalente est essentielle pour produire un effet synergique. Cela suggère un concept par lequel une molécule de spécificité relativement faible (le peptide sulfaté), couplé à un composé hautement spécifique (le mCD4) peut atteindre des affinités très élevées pour sa cible. Ce composé cible donc avec succès deux domaines critiques et hautement conservés dans la gp120 de manière corécepteur indépendante. Dans des cultures de cellules sanguines il inhibe la réplication de souches de VIH-1 adaptées au laboratoire Ba-L (tropisme R5) et LAI (tropisme X4), (pour laquelle il n'existe aucun inhibiteur antagoniste efficace avec IC_{50} aussi bas que 1 nM. Le mCD4-P3YSO$_3$ inhibe également l'entrée du virus primaire (sous-type A, B et C).

6.2 Preface

As shown in Chapter 5, the interaction system with solubilized coreceptors has been setup and validated with the study of the CXCL12 isoforms. Once the coreceptors are solubilized, they retain their functionality when immobilized on the biacore surface and kinetic information can be obtained between the coreceptors and the chemokine isoforms which corresponds to affinity data obtained in cell-based assays. We thus were confident that the coreceptor surfaces would be functional and ready to be used in the assay to screen HIV-1 entry inhibitors.

Navratilova *et al.,* have set-up a similar technique, whereby CCR5 coreceptors are solubilized and they demonstrate CCR5 utilizing envelopes binding to the immobilized CCR5 coreceptors (Navratilova, Sodroski et al. 2005; Navratilova, Dioszegi et al. 2006). However, an interaction between CXCR4 utilizing envelopes and surface captured isolated CXCR4 has never been reported.

6.3 Introduction and Preliminary approach

Almost 30 years after the discovery of human immunodeficiency virus – 1 (HIV-1), 33 million people globally are infected of which 68% reside in Sub-Saharan Africa, and there is still no cure. However, the virus is *not* eradicated and treatment interruption does occur which is the cause of the outgrowth of drug-resistant viruses. In addition to treatment adherence issues, the long-term adverse side-effects as well as the exorbitant financial burden of these therapies are counteractive forces especially in developing countries where the epidemics are the worst (UNAIDS 2011).

Targeting HIV-1 entry has recently attracted a lot of attention with already two drugs approved and licensed by the food and drug administration (FDA); Enfuvirtide [fuzeon] (Matthews, Salgo et al. 2004) and Maraviroc [Selzentry] (Dorr, Westby et al. 2005). This stage of the viral life-cycle is particularly attractive as when the viral envelope (env) approaches the target $CD4^+$ T cell, the primary receptor is engaged (CD4) (Klatzmann, Champagne et al. 1984) and a series of conformational changes occur in env which expose/create the cryptic coreceptor binding domain, which is briefly exposed before docking onto either coreceptor (CCR5 or CXCR4) (Alkhatib, Combadiere et al. 1996; Feng, Broder et al. 1996). This cryptic domain is composed of a four-stranded β-sheet, referred to as the CD4-induced domain (CD4i) and in conjunction with the V3 loop, they play vital roles in coreceptor binding (Rizzuto, Wyatt et al. 1998; Hartley, Klasse et al. 2005).

In the early stages of infection, HIV-1 is transmitted by CCR5-utilizing viruses (R5) which infect macrophages, however, as the disease progresses and the immune system is further compromised, more virulent variants emerge which are CXCR4-utilizing viruses (X4 or R5X4) that infect T-cells (Connor, Sheridan et al. 1997). Before HIV-1 engages the host CD4 and coreceptor molecules, GAGs concentrate the virus on the cell surface and aid to sequester and concentrate the virus near its receptors (Roderiquez, Oravecz et al. 1995; Mondor, Ugolini et al. 1998). Recently, a phenomenal study has shown that when a synthetic 12mer

GAG oligosaccharide is covalently linked to a CD4 mimetic peptide (mCD4), the mCD4 binds to env, triggering the conformational change necessary to expose the CD4i which then permits the covalently attached 12mer GAG to bind the CD4i pocket and effectively block both R5 and X4 HIV-1 entry with nM activity (Baleux, Loureiro-Morais et al. 2009). This dodecasaccharide is fully sulphated (18 sulphates) and is extremely difficult and complex to synthesize. In order to perform structure-functional analyses, a large quantity of known sequence would be required, thus synthesizing the GAG fragment is almost impossible.

Glycosaminoglycans are extremely complex molecules; their basic unit is a disaccharide of which there are 48 different variations (depending on sulphation modifications and COO^- epimerisations etc.). Thus if we were to calculate the number of different variations for a 12mer, we would reach up to over 10 billion possible sequences, a staggering degree of variation on which it is thus impossible to perform structure-function analysis. One approach to screen for a HS_{12}mer with the optimal sulphation sequence required for CD4i binding and entry inhibition, would be to screen a bank of differently sulphated HS_{12}mers.

6.3.1 Generation of an HS_{12} differently sulphate Library

Heparin (HP) dodecasaccharides were prepared by R.Sadir as previously described (Sadir, Baleux et al. 2001). Briefly, porcine mucosal HP (10g) was depolymerized with heparinase I and the digested mixture was resolved from di-(dp2) to octa-(dp18) decasaccharide on a Bio-Gel P-10 column. The eluted material was detected by absorbency at 232 nm and dp2 up until dp12 were further purified by strong anion-exchange HPLC, dialyzed against distilled water, and quantified either by a colorimetric assay or weighed.

The inhibitory action of the oligosaccharide domain of the mCD4-HS_{12} molecule needed to be further studied and optimised in order to improve its specificity and affinity. For this purpose, we prepared an HS_{12} derived molecular library (containing 12 initial sub-populations of differently sulphated HS_{12}mers). This was obtained by fractionating the HP12 (dodecasaccharide) fraction (obtained from the above mentioned depolymerised HP fractionation) under a stong anionic gradient on the HPLC (Figure 6.1 A) .The sub-populations were grouped into 12 larger sub-populations, with each group containing HS_{12} molecules with presumably similar overall charges, however, within each sub-population there are many different species with an unequal organisation of sulphate molecules.

This process is also very time consuming and a low level of purity of each sub-population of HS_{12} is obtained due to the highly complex and heterogeneous nature of heparan sulphate. A homogeneous population would be almost impossible to obtain (Figure 6.1), therefore, this technique was determined to be too difficult and unable to isolate a significant amount of a pure molecule.

For this reason, it is highly advantageous to use peptide GAG-mimetics, As a result, our collaborators have synthesized HS mimetics that possess carboxyl, hydroxyl and sulphate groups, which mimic those found on an oligosaccharide. These GAG mimetics were also screened for their inhibitory capacity of the gp120-CD4–coreceptor interaction, using the recently set-up and optimised interaction system on the SPR platform.

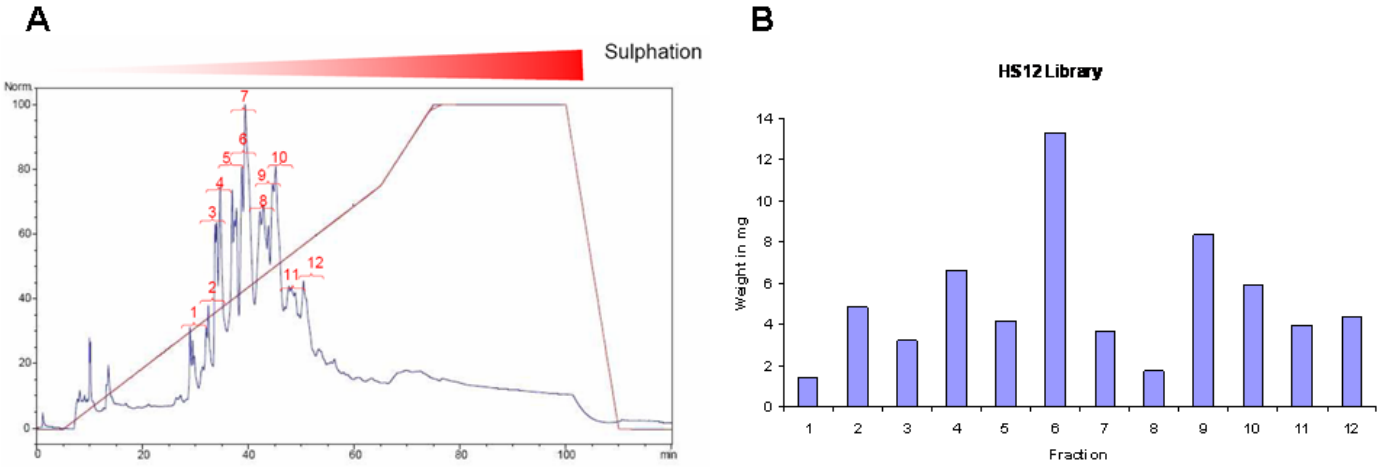


Figure 6.1 A Typical HPLC elution profile of HS_{12} showing absorbance at 232nm as a function of time (min) and B a histogram corresponding to the quantities (in mg) of each separated HS_{12} library fraction.

R5 viruses enter their target cells through the recognition and binding of the N – terminus and the second extracellular loop (ECL) of CCR5 (Rucker, Samson et al. 1996; Farzan, Choe et al. 1998; Cormier, Persuh et al. 2000). Huang *et al.,* showed that two sulphated tyrosines (at positions 10 and 14) in the N-terminus of CCR5 interact within a binding pocket on gp120 formed between the base of the V3 loop and the bridging sheet (Huang, Lam et al. 2007) and salt bridges are made with basic residues in this pocket. Interestingly, sulphotyrosines are also found in the N-terminal of CXCR4, as well as in the heavy chain 3rd complementary determining region (CDR H3) of CD4i HIV-1 neutralizing monoclonal antibodies 412d and E51 (Choe, Li et al. 2003; Huang, Venturi et al. 2004). Thus, tyrosine sulphation is a post-translational modification that plays a critical role in protein-protein interactions and nature has managed to mimic these modifications in order to attempt to inhibit certain viruses.

We thus hypothesized, that due to the fact that peptide synthesis is much simpler compared to glycosaminoglycan synthesis (practically speaking), and a peptide would be much more amenable to structure-function analysis due to its known and homogenous sequences, we produced five peptides (13 amino acids in length) each conjugated to mCD4 and containing amino acids that mimic the OH^-, COO^- and $SO4^{3-}$ residues found in GAGs. The peptide containing 6 sulphated tyrosines not only mimics the sulphate residues in GAGs but also mimics those found in the N-terminal of both CCR5 and CXCR4. Since the neutralizing antibodies (E51 and 412d) are induced in patients against the CD4i, their existence proves that targeting the cryptic CD4i site with peptides containing sulphotyrosines is a valid strategy for HIV-1 inhibition (DeVico 2007). Additionally, peptides are more likely to access the small cryptic CD4i site, compared to large cumbersome antibodies. The following base sequence was used for peptide synthesis: S(XDXS)n where X stands for the different amino acids and n = 3 (the total length equivalent to that of a GAG 12mer). Peptide PY3 (where X = non-sulphated tyrosine), $P3YSO_3$ (where X = sulphotyrosine), P3Asu (where X =

aminosuberic acid), P3pF (where X = p-carboxymethyl phenylalanine) and E13 (where the entire length is glutamic acid – a non-specific polyanion) were synthesized by our collaborators.

In order to test the inhibitory capacity of these peptide GAG-mimetic inhibitors, we aimed to assess their capacity to inhibit gp120-CD4 from binding to the solubilized immobilized coreceptors (CCR5 and CXCR4). The optimization of the surface plasmon resonance (SPR) technique, whereby solubilized coreceptors were immobilized on a sensorchip and interactions between their binding ligands were monitored in a complex buffer, was performed as described in Chapter 5 for CXCR4, and was developed for CCR5 as described in this Chapter. Thus, we used this technique to screen the different peptides for their capacity to inhibit gp120 (either commercial MN [X4] or YU2 [R5]) -CD4 complexes from binding to their respective coreceptors, CXCR4 or CCR5. We report affinities of the gp120-CD4 complexes for their respective coreceptors that equal those reported in other studies using either similar or cell-based and proteoliposome-based techniques (Doranz, Orsini et al. 1999; Babcock, Mirzabekov et al. 2001; Navratilova, Sodroski et al. 2005). The sulphotyrosine containing peptide conjugated to mCD4 (mCD4-P_3YSO_3) demonstrated the highest success in inhibiting gp120-CD4 complexes from binding to the coreceptors and was thus further evaluated in viral entry inhibition using peripheral blood mononuclear cell infection assays. This molecule inhibits both R5 and X4 viruses with nM activity and is not toxic for the cells up to 1μM, unlike most current CCR5 and CXCR4 antagonists. This novel entry inhibitor targets the virus and not the host; thus further reducing the risk for host-toxicity issues.

6.4 Results

We aimed to asses whether the solubilized coreceptors (CCR5 and CXCR4) were capable of recognising their gp120 ligands in an immunoprecipitation experiment. Here, protein G sepharose beads were coupled to the 1D4 antibody which binds to the C9 tag at the C-terminal of CCR5/CXCR4 and purified it from the mass of solubilized membrane proteins. Ligands (gp120, antibodies) were immunoprecipitated by the bound coreceptors in the absence and presence of various inhibitors. The presence of the ligands was determined by antibody binding and western blot. These experiments were not sufficiently conclusive and would required futher optimization, we thus directly used the SPR approach as described on chapter 5.

6.4.1 Surface Plasmon Resonance Screening platform

We thus commenced the screening process of the various inhibitors (HS_{12} fractionated library and peptide GAG mimetics) over the following surfaces prepared on the biacore: 17b, biotinylated HS, mCD4 / full length CD4 or solubilized CCR5 / CXCR4 (Figure 6.2). For all the surfaces, except that of the solubilized coreceptors, a surface prepared with streptavidin served as the negative surface foreseen for background binding subtraction. In the case for the GPCR surfaces (Figure 6.2, scenario D), the 1D4 antibody served as the negative surface. Since 17b has been used by many previous studies as a surrogate

coreceptor, we included a 17b surface in our screening assays, however this mAb only partially covers the entire coreceptor binding domain. The coreceptor binding site is constituted not only by the bridging sheet but also by the V3 loop (Dragic 2001; Baleux, Loureiro-Morais et al. 2009; Dervillez, Klaukien et al. 2010) and it is for this reason that we decided to use native solubilized coreceptors to immobilize on the biacore surface for our binding studies.

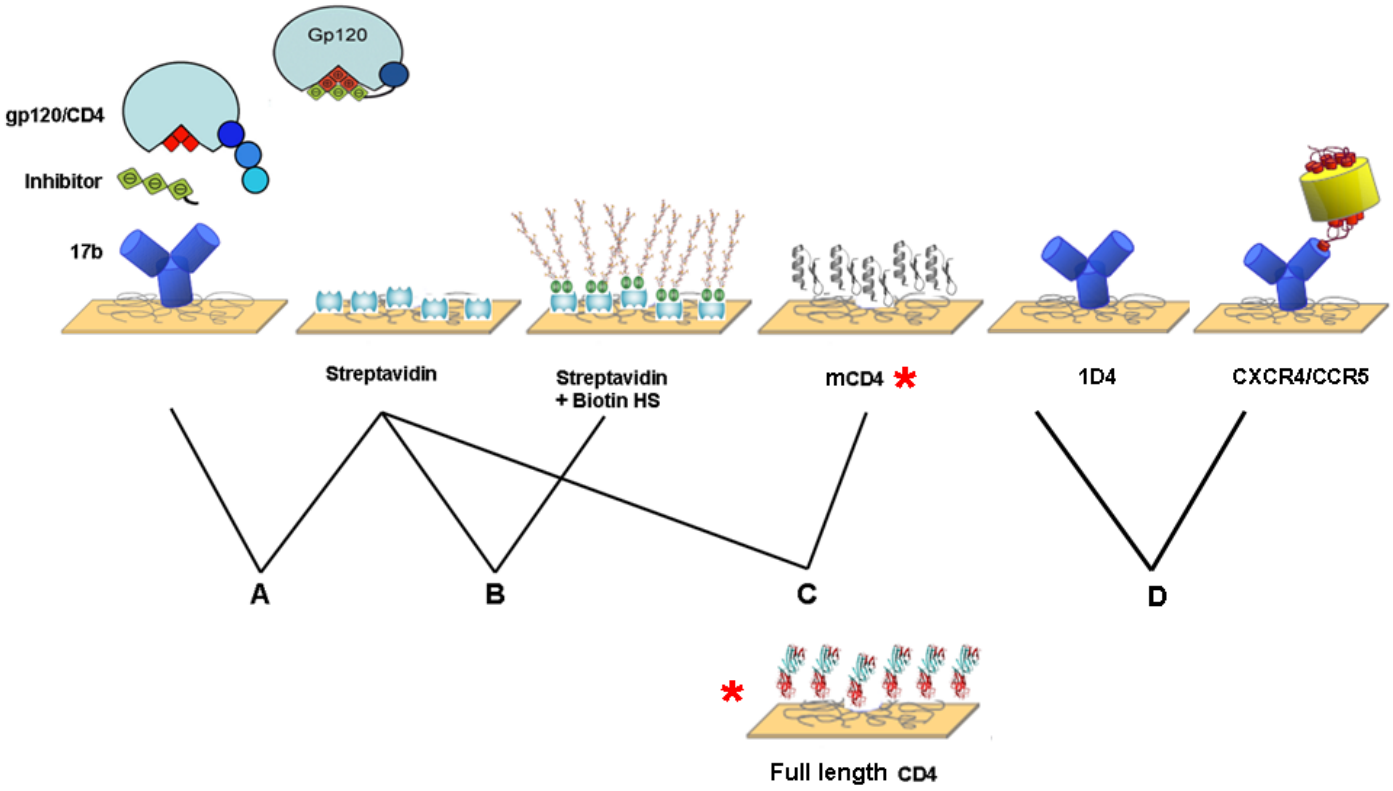


Figure 6.2 Schematic representing the four different scenarios which were used when screening the various entry inhibitor molecules. Scenario A, B and C use streptavidin as a reference surface and scenario uses 1D4. Scenarios A has 17b as the test surface, B has biotinylated Heparan Sulphate (HSb), C has either full length CD4 or mCD4 and D has either CCR5 or CXCR4 solubilized coreceptors. For scenario C, either mCD4 or full length CD4 was immobilized and will be indicated in the text.

Throughout the experiments, laboratory adapted and expressed gp120 MN (CXCR4 utilizing envelope) and primary isolate gp120 YU2 (CCR5 utilizing envelope) were used when screening for molecules that inhibited gp120-CD4 from binding to the immobilized coreceptors. Before any inhibitory molecules were screened, conformationally dependent monoclonal antibodies (12G5, 4G10 and 2D7, N-terminal antibody) were injected over the immobilized coreceptors to detect whether or not they recognised their respective coreceptors (CXCR4 and CCR5) (Figure 6.3). Together with the binding studies described in Chapter 5 between CXCL12 and CXCR4, we were very confident that our solubilized coreceptors were functional and suitable for the screening studies for the entry inhibitors.

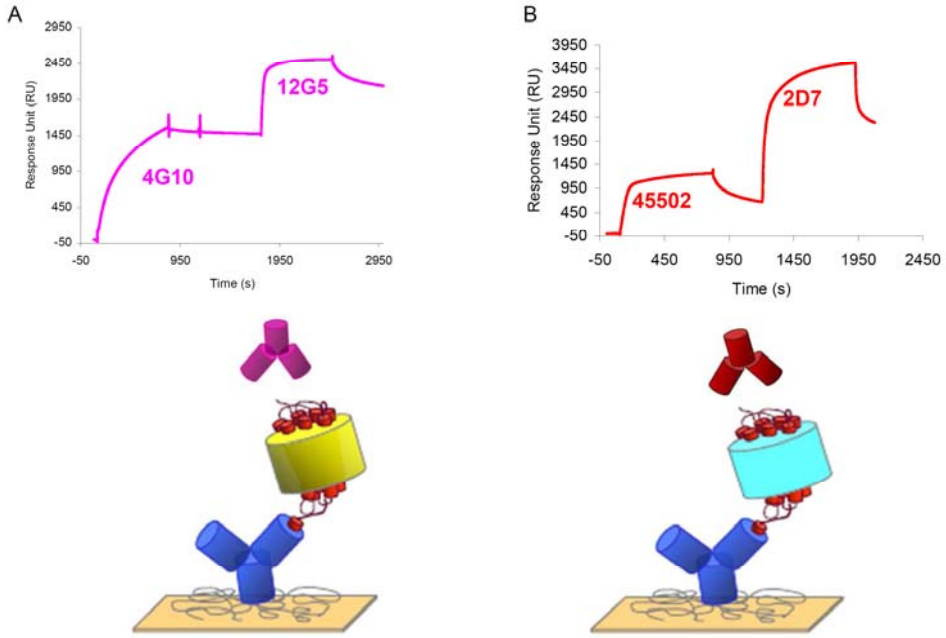


Figure 6.3 Binding of conformational sensitive (12G5 and 2D7) and non structural dependant antibodies (4G10 and 45502) onto solubilized CXCR4 (A) and CCR5 (B) respectively immobilized onto CD4 sensor chips through the high affinity interaction with 1D4. Cartoon diagrams represent the different coreceptors immobilized on the sensor chip surface.

6.4.2 A synthetic heparan sulfate-mimetic peptide conjugated to a mini CD4 displays very high anti-HIV-1 activity independently of coreceptor usage

This part of the thesis work has been accepted for publication and thus will be inserted here as a 'manuscript'.

A synthetic heparan sulfate-mimetic peptide conjugated to a mini CD4 displays very high anti-HIV-1 activity independently of coreceptor usage

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Running title: Novel gp120 targeting HIV-1 entry inhibitor

SUMMARY

The HIV-1 envelope gp120, which features both the virus receptor (CD4) and coreceptor (CCR5/CXCR4) binding sites, offers multiple sites for therapeutic intervention. However the latter becomes exposed, thus vulnerable to inhibition, only transiently when the virus has already bound cellular CD4. To pierce this defense mechanism, we engineered a series of heparan sulfate mimicking tridecapeptides and showed that one of them target the gp120 coreceptor binding site with μM affinity. Covalently linked to a CD4-mimetic which binds to gp120 and renders the coreceptor binding domain available to be targeted, the conjugated tridecapeptide now displays nM affinity for its target. Using solubilized coreceptors captured on top of sensorchip we show that it inhibits gp120 binding to both CCR5 and CXCR4 and in peripheral blood mononuclear cells broadly inhibits HIV-1 replication with an IC_{50} of 1 nM.

Highlights:

- The HIV coreceptors, CCR5 and CXCR4, were functionally captured on sensor surfaces
- Heparan sulfate mimetic peptides $S(XDXS)_3$ target the gp120 coreceptor binding site
- Covalently linked to a CD4 mimetic they block gp120 binding to both CCR5 and CXCR4

A mCD4-$S(XDXS)_3$ conjugate inhibits HIV replication with an IC_{50} of 1 nM

INTRODUCTION

Although tremendous progress has been made in the development of antiviral drugs to treat human immunodeficiency virus (HIV-1) infection (De Clercq 2007) and despite the availability of some 25 approved antiretroviral compounds (most of which target HIV-1 enzymes) the virus continues to be a major concern and remains one of the leading causes of death worldwide. The rapid emergence of drug-resistant viral strains, the inability of current therapy to completely eradicate the virus and the strong adverse side effects associated with their long-term use (Shafer and Schapiro 2008) compromise treatment in patients benefiting from these therapies, and make the development of new therapeutic options of utmost importance (Flexner 2007). Inhibition of HIV-1 entry, a process based on the sequential interaction of the viral glycoprotein (gp120) with the cell surface CD4 (Klatzmann, Champagne et al. 1984) and either one of the two chemokine receptors CCR5 or CXCR4 (Alkhatib, Combadiere et al. 1996; Feng, Broder et al. 1996), holds particular promise in addressing complications of current therapy and has become a compelling target for controlling viral replication (Tilton and Doms 2010). The recent approval of maraviroc, a CCR5 antagonist (Maeda, Nakata et al. 2004; Dorr, Westby et al. 2005), has validated entry inhibition as a viable approach. However, to avoid the selection of pre-existing and more pathogenic CXCR4-using HIV-1 (for which no effective antagonistic inhibitors yet exist) maraviroc has been licensed for the treatment of patients infected with viral strains using CCR5 only.

On the virus side, the gp120 constitutes the central element for all interactive events occurring during the pre-entry steps. A wealth of evidence has shown that gp120 binding to CD4 not only permits virus attachment, but also triggers extensive conformational changes of the envelope that fold and/or expose a four-stranded β-sheet, known as the CD4-induced (CD4i) domain (Wu, Gerard et al. 1996). Being critically involved in CCR5/CXCR4 recognition and

highly conserved, this domain represents an attractive pharmacological target. Although inhibition of protein–protein interactions is clearly challenging, a striking feature of the CD4i domain is its basic nature (Kwong, Wyatt et al. 1998; Rizzuto, Wyatt et al. 1998) and, not surprisingly, many of this domain's ligands are characteristically acidic. This includes peptides selected by phage display screening (Dervillez, Klaukien et al. 2010), sulfated oligosaccharides from the heparan sulfate (HS) family (Vives, Imberty et al. 2005; Crublet, Andrieu et al. 2008), aptamers (Cohen, Forzan et al. 2008), peptides derived from neutralizing antibodies (Dorfman, Moore et al. 2006), compounds issued from *in silico* screening of molecular libraries (Acharya, Dogo-Isonagie et al.) or peptides derived from the N-terminal sequence of CCR5 itself which comprise sulfotyrosines importantly contributing to gp120 binding (Cormier, Persuh et al. 2000; Farzan, Vasilieva et al. 2000). The cryptic nature of this CD4i surface prior to CD4 binding however limits its accessibility both temporally and spatially, and makes it a relatively intractable pharmacological target. In that context, we recently developed a new class of compounds, in which a CD4 mimetic peptide (mCD4) was linked to a HS dodecasaccharide (HS_{12}) and showed that mCD4 exposed the gp120 CD4i domain and renders it available to be blocked by the HS_{12} oligosaccharide (Baleux, Loureiro-Morais et al. 2009).

Here, to further develop this concept we engineered a series of tridecapeptides that mimic HS, the synthesis of which, although amenable to large scale production, remains extraordinary complex (Dilhas, Lucas et al. 2008). We then set up a binding assay in which detergent solubilized CCR5 and CXCR4 were both functionally captured on top of sensorchips and used them to show that, conjugated to a mini CD4, a HS mimicking peptide efficiently targets the CD4i domain of gp120 and blocks its interaction with the coreceptors. This compound displays antiviral activity against LAI and Ba-L HIV strains with an IC_{50} as low as 1 nM, two to four orders of magnitude lower than the above described anionic compounds. To our

knowledge this is the most potent gp120 targeting molecule, with the unique property to simultaneously block two critical and conserved regions of gp120. Importantly it inhibits CCR5 and CXCR4 using viruses equally well, and is also highly active against a number of viral primary clinical isolates. These results should have strong implications for the development of a new anti-HIV-1 therapy.

RESULTS

HIV-1 coreceptors immobilisation and gp120 binding

Assessing the ability of molecules to target the coreceptor binding site of gp120 would strongly benefit from a direct coreceptor-gp120 interaction assay. To that end, both HIV-1 coreceptors were solubilized from Cf2Th cells, recombinantly expressing either CCR5 or CXCR4, using a specific cocktail of lipids and detergents that was adapted from that previously described (Navratilova, Sodroski et al. 2005). Solubilized coreceptors, which feature a C-terminal C9 tag (Mirzabekov, Bannert et al. 1999; Babcock, Mirzabekov et al. 2001) allowing their oriented capture with the cognate 1D4 antibody, were immobilized on top of a sensorchip to a level of ~ 4000 resonance units (RU). To verify whether the coreceptors remained functional we first investigated their binding capacity with the conformationally sensitive mAb 2D7 for CCR5 (Lee, Sharron et al. 1999; Khurana, Kennedy et al. 2005) and 12G5 for CXCR4 (Baribaud, Edwards et al. 2001). As shown in Figure 1AB, injection of these mAbs over the CCR5 and CXCR4 functionalized surfaces gave rise to strong and coreceptor specific binding signals indicating both the presence of the coreceptor on the surface, and the integrity of the corresponding epitopes.

Following this, we analyzed whether the immobilized coreceptors bound gp120, in a CD4 dependant manner. For that purpose, 100 nM of either YU2 or MN (R5 and X4 envelopes respectively), in the absence or presence of mCD4, a CD4 mimetic peptide that was previously found to bind gp120 and induce the conformational change that lead to the folding/exposure of the coreceptor binding site (Baleux, Loureiro-Morais et al. 2009), were injected over the coreceptor surfaces. Both envelopes interacted with their coreceptors, presumably because the CD4i epitope is transiently exposed on the dynamic structure of

gp120, as already observed with anti-CD4i antibodies (Thali, Moore et al. 1993). The binding responses, however, were significantly enhanced by the presence of mCD4, and efficiently inhibited by 1 μM of maraviroc or AMD3100 (Figure 1CD), two compounds targeting CCR5 and CXCR4 respectively, and having anti-HIV-1 activity (Tilton and Doms 2010). Next, dose response experiments were performed with mCD4:gp120 ratios fixed at 1:1, and injected over the immobilized CCR5 or CXCR4 surfaces. Sensorgrams were obtained for both envelopes (Figure 1EF), which evaluations (see supplemental experimental procedures) returned estimated affinities of 11.5 ± 2.9 nM and 154 ± 68 nM for CCR5 and CXCR4 respectively. These values were identical to that reported by a similar technique (Navratilova, Sodroski et al. 2005) or radioligand binding assay with cell membrane-embedded CCR5 (Doranz, Baik et al. 1999) as to that reported for CXCR4, using proteoliposome embedded coreceptors and radiolabelled gp120 (Babcock, Mirzabekov et al. 2001).

We previously reported that the gp120 CD4i epitope can be targeted by HS (Vives, Imberty et al. 2005; Crublet, Andrieu et al. 2008), and that a HS dodecasaccharide covalently linked to mCD4 (mCD4-HS_{12}) binds gp120 and blocks its subsequent interaction with mAb 17b (Baleux, Loureiro-Morais et al. 2009). MAb 17b belongs to a group known as “anti-CD4i” antibodies, which recognizes a conserved element of gp120, induced by CD4 and partially overlapping the coreceptor binding site (Xiang, Doka et al. 2002). We thus made use of the coreceptor binding assay described above to investigate whether mCD4-HS_{12} would also inhibit gp120 binding to CCR5 and CXCR4. As shown in Figure 1GH, both YU2 and MN gp120 in complex with mCD4-HS_{12} featured a strongly reduced ability to recognize CCR5 or CXCR4 compared to that of gp120 in complex with mCD4 alone. This suggests that such molecules could serve as lead compounds for the future development of a new class of entry inhibitors.

Chemical synthesis of mCD4 linked HS mimetic peptides

HS are however notoriously difficult to synthesize. In addition, their inherent sequence heterogeneity, in terms of sulfation pattern and saccharide composition, would currently make the preparation of a dodecamer series out of reach. Thus, based on the mCD4-HS_{12} template, we tested the hypothesis that the HS moiety could be mimicked by peptides, the chemical synthesis of which is more straightforward, and more easily amenable to sequence-activity relationship investigation. To display the functional hydroxyl, carboxyl and sulfate groups that characterize HS, peptides comprising Ser, Asp, and Tyr, the latter being possibly sulfated, were considered. This strategy is supported by the observation that a SYDY tetrapeptide binds to the HS binding domain of the vascular endothelial growth factor (Maynard and Hubbell 2005) and that phage display screenings against the CD4i epitope of gp120 returned sequences enriched in YD motifs (Dervillez, Klaukien et al. 2010). It is also worth noting that a number of antibodies against the gp120 coreceptor binding domains feature sulfotyrosines in their paratope, as does the N-terminus of both CCR5 and CXCR4 (Choe, Li et al. 2003).

Building of a $S(XDXS)_n$ sequence (were X stands for different possible amino acids - see below) using the peptide builder of Hyperchem 5, showed that a 13 amino acid peptide (n = 3), in its extended configuration (φ, ψ and ω angles set to 180 °) would have a length equivalent to the HS 12 mer (data not shown). Thus a tridecapeptide, alternating OH/COO^- and OH/SO_3^- groups, having the sequence: $SY_{SO3}DY_{SO3}SY_{SO3}DY_{SO3}SY_{SO3}DY_{SO3}S$ (X being in this case a sulfotyrosine; Y_{SO3}) was first synthesized ($P3YSO_3$). The non sulfated equivalent (P3Y) was also prepared along with a number of other peptides in which X was replaced by p-carboxymethyl phenylalanine (P3pF) or aminosuberic acid (P3Asu), two residues that have been shown to functionally mimic sulfotyrosine in cholecystokinin type B receptor ligand CCK8 (McCort-Tranchepain, Ficheux et al. 1992) and sulfakinins (Nachman, Vercammen et al. 2005). A tridecaglutamate (displaying 13 carboxylic groups) was also

prepared (E13) as a non specific poly anionic peptide (Figure 2). In order to maintain an appropriate distance between mCD4 and these peptides, enabling the final molecule to reach both the CD4 and coreceptor binding sites, a γ-aminobutyric acid (γ-Abu) was introduced on their N-terminus. These peptides were derivatized with S-acetylthiopropionic acid to allow the coupling to Lys^5 of a maleimide-activated mCD4. All compounds were purified to a level of 95% by RP-HPLC (see Tables S1 and Figures S1, S2), controlled by mass spectrometry and quantified by amino acid analysis as described in the supplemental experimental procedures.

mCD4 linked HS mimetic peptides inhibit binding of gp120 to CD4, mAb 17b and coreceptors

To verify that peptide conjugation did not prevent the ability of mCD4 to interact with gp120, a competition assay was performed, in which YU2 or MN were incubated with the different mCD4 conjugates and injected over a CD4 functionalized surface. Results showed that the mCD4-conjugates all very efficiently prevent gp120-CD4 interaction, with greater potency than that of unconjugated mCD4 (Figure 3AB). Next, the capacity of the anionic peptides to target the gp120 CD4i epitope was investigated by analyzing their ability to prevent gp120 binding to mAb 17b, in the presence of soluble mCD4. While unliganded gp120 was not (MN) or only poorly (YU2) recognized by mAb 17b (Figure 3 CD; blue trace), preincubation with mCD4 strongly promoted binding (black trace). When the gp120-mCD4 complexes were further incubated with 5 μM of the above described tridecapeptides, strong inhibition was observed for $P3YSO_3$ (green trace). The tridecaglutamate (E13) was devoid of activity, indicating that the anionic character of the peptide is not sufficient to provide binding, as were the unsulfated P3Y or the sequence in which the sulfotyrosine mimetics (pF and Asu) were introduced (Figure 3CD; black traces). HS_{12} (red trace) also fully blocked mAb17b binding to

MN-, but not to YU2- gp120. Together, this showed that amongst the different peptides investigated only the $SY_{SO3}DY_{SO3}$ motif competes with mAb 17b to interact with the gp120 CD4i domain. To better quantify the inhibitory activity of this peptide, the same assay was run, with a range of $P3YSO_3$ concentrations, and compared with HS_{12}. A similar concentration dependency was observed on both R5 (YU2) and X4 (MN) envelopes, with IC_{50} of 2.9 and 3.1 μM respectively indicating that, interestingly, $P3YSO_3$ interacts with gp120 independently of coreceptor tropism. In contrast, HS_{12} strongly inhibited the interaction between MN and mAb 17b (with a concentration as low as 0.5 μM) but was ineffective towards YU2, at concentrations up to 10 μM (Figure 3EF). Next, to determine the binding mechanism of the mCD4-$S(XDXS)_3$ constructs, X4- and R5- gp120 were immobilized on a sensorchip and first allowed to bind to mCD4, mCD4-P3Y or mCD4-$P3YSO_3$. The resulting complexes were then probed with mAb 17b, the binding of which being a marker of the coreceptor binding site accessibility. As expected, mCD4 binding to gp120 renders the coreceptor binding site accessible, a point that was also observed, although with a lower efficiency, with mCD4-P3Y. These data indicate that while mCD4-P3Y bound to gp120, the unsulfated peptide did not sufficiently interact with the newly available surface to block mAb 17b recognition. In contrast, when mCD4-$P3YSO_3$ was used instead of mCD4 or mCD4-P3Y, the mAb 17b was no longer able to interact with the complex. Altogether, these data thus support the view that mCD4 first binds to gp120 and exposes the coreceptor binding site, with which the $P3YSO_3$ moiety then interacts strongly enough to prevent antibody binding (Figure 3GH). Finally, using the direct gp120-coreceptor interaction assay described in Figure 1, we also demonstrated that mCD4-$P3YSO_3$ very potently inhibits gp120 binding to both CCR5 and CXCR4 (Figure 3IJ). This suggests that this compound could be a coreceptor independent HIV-1 entry inhibitor.

mCD4 linked P3YSO$_3$ peptides display strong antiviral activity

Having characterized the binding mechanism of these compounds, we investigated whether these anionic peptides, either conjugated or not to mCD4 displayed anti-HIV-1 activity. This was performed using an assay in which viral replication was measured (reverse transcriptase quantification) in the supernatant of blasted peripheral blood mononuclear cells (PBMC) isolated from three to four donors, and infected by either of the HIV-1 reference strains R5 (Ba-L) or X4 (LAI). When used alone, none of the peptides demonstrated antiviral activity at the highest concentration tested (500 nM; data not shown). However, when conjugated to mCD4, they displayed inhibitory activity against the LAI strain, with effective doses giving 50% inhibition (ED_{50}) as low as 0.5 nM for mCD4-P3YSO$_3$, which compares well to 1.4 nM for mCD4-HS$_{12}$. Consistently with the biochemical data, the importance of the sulfate groups was shown by the large increase of ED_{50} (98 nM) that characterized mCD4-P3Y, while the other anionic peptides (mCD4-P3pF, mCD4-P3Asu and mCD4-E13) displayed 8.2 to 30 nM ED_{50} (Figure 4A). The Ba-L strain was also very strongly inhibited by mCD4-P3YSO$_3$, with an ED_{50} of 1.3 nM, versus 18 nM for mCD4-HS$_{12}$. None of the other conjugates displayed significant antiviral activity (Figure 4B). AZT, used as a reference anti-HIV molecule in the same assay returned ED_{50} of 8.7 and 11 nM for R5 and X4 viruses respectively (Figure 4AB).

We also observed that mCD4-P3YSO$_3$ does not need to be preincubated with the virus to be active. Indeed, addition of the molecule either to the cells, prior to the viral challenge or to the virus prior to the cell infection return, identical results (supplementary table S2). This is consistent with the high affinity this molecule displays for the viral envelope, presumably enabling a fast binding to its target, and also suggests a potential use of this kind of compounds as a microbicide, a condition in which inhibitors are present within the host tissues, before viral infection.

Having established that mCD4-P3YSO$_3$ displayed very strong antiviral activity against LAI and Ba-L HIV-1 strains, used as model systems, we extended our investigations to using a series of more clinically relevant primary strains, including 92UG029, SF162, 92US723, 96USHIPS4, 92HT599 and 98IN017. As shown in Table 1, mCD4-P3YSO$_3$ displayed a high level of antiviral activity, characterized by ED_{50} in the range of 0.2 to 1.2 nM for five of them, and 29 nM for HIV-1 98IN017. As for the LAI and Ba-L strains, the mCD4 or P3YSO$_3$ were only poorly- or in- active, further supporting the very strong synergistic effect induced by the coupling strategy. None of the molecules showed cytotoxicity at up to 1 μM (data not shown).

DISCUSSION

Targeting gp120 for HIV-1 inhibition is both attractive (because the protein engages multiple interactions key to viral entry, thus offering multiple sites for inhibition) and challenging (in the entry complex, the buried surface to block comprises both the gp120-CD4 and gp120-coreceptor interfaces). Although protein-protein interfaces are often relatively featureless and devoid of traditional cavities into which a small molecule can dock, the realization that the gp120 coreceptor binding site displays a restricted number of functionally important basic residues has very recently attracted the attention of many studies. Many of them reported that anionic molecules target the CD4i epitope, as shown by their ability to competitively inhibit mAb 17b binding with IC_{50} in the 1-100 μM range (Cormier, Persuh et al. 2000; Farzan, Vasilieva et al. 2000; Cohen, Forzan et al. 2008; Crublet, Andrieu et al. 2008; Brower, Schon et al. 2009; Dervillez, Klaukien et al. 2010; Seitz, Rusert et al. 2010; Acharya, Dogo-Isonagie et al.; Kwong, Dorfman et al. 2011). HS belongs to this class of CD4i domain targeting molecules (Crublet, Andrieu et al. 2008), and a highly sulfated and regular sequence comprising 12 monosaccharide units has been recently prepared. Conjugated to mCD4, it displays strong anti-HIV-1 activity (Baleux, Loureiro-Morais et al. 2009). However, HS is extraordinary complex and heterogeneous in sequence (Esko and Lindahl 2001). Based on the 48 different units that the polymer theoretically comprises, a 12 mer library would reach 10^{10} molecules. Although the reality is less (all the combinations are not possible), it remains much more than can be realistically synthesized for structure-activity relationship studies. Thus, to further develop this kind of molecule we attempted to design HS mimetic peptides, with the general sequence $S(XDXS)_3$ and showed that when X was a sulfotyrosine, it binds to the CD4i epitope, blocking mAb 17b with IC_{50} of 3 μM, thus comparing very well with the above mentioned molecules. Interestingly, this peptide interacts equally well with R5 and X4 gp120, while HS especially binds to the X4 envelope (Figure 3EF). More importantly, the

conjugation of this peptide to mCD4 dramatically enhances its binding activity, the conjugated molecule being able to fully prevent the gp120/mAb 17b interaction at low nM concentration, showing that the covalent linkage induced a strong synergistic effect. This is consistent with the view that high affinity mCD4 binding takes place initially, inducing the exposure of the mAb 17b epitope to which the sulfated peptide can then bind. As such this molecule is distinct from other mAb 17b blocking peptides that suppress CD4 binding and subsequent coreceptor binding site exposure through an allosteric inhibitory effect rather than competitive inhibition (Biorn, Cocklin et al. 2004).

Although widely used as a CCR5 or CXCR4 surrogate, mAb 17b however only imperfectly defines the gp120 coreceptor binding site which, in addition to the CD4 induced bridging sheet, is also constituted by the V3 loop in particular (Dragic 2001). Thus, to better asses the blocking efficiency of molecules targeting the gp120-coreceptor interaction, and taking into account domains outside the CD4i epitope itself, CCR5 and CXCR4 were solubilized and functionally captured on top of biacore sensorchips. Binding of gp120 to CCR5 and CXCR4 proved to be both CD4 and concentration dependent and inhibited by specific antagonists. Fitting of the binding data was expectedly complicated by several parameters, such as the complexity of the buffer system used, the reversible nature of both the 1D4-coreceptor and mCD4-gp120 complexes and the conformational flexibility of gp120, thus the calculated affinity values reported should probably be considered as estimates only. Nevertheless, we report K_D's of 10 and 150 nM for the YU2-CCR5 and MN-CXCR4 interactions respectively, comparable to those obtained with cellular systems in which the coreceptors remained in their natural cell membrane environment (Doranz, Baik et al. 1999; Babcock, Mirzabekov et al. 2001). This assay provides a useful, label free method, to identify both binding capacity of envelopes and inhibitory activity of potential drugs. This was especially true in the framework of this study investigating sulfated/polyanionic compounds to target the gp120 coreceptor

binding site. Although tyrosine sulfation of coreceptors has been shown to play a less significant role in CXCR4- than in CCR5-dependent HIV-1 entry (Farzan, Babcock et al. 2002), we found that when conjugated to mCD4 the sulfated $P3YSO_3$ displays very strong binding activity toward both R5- and X4- gp120. Using this assay, we indeed report that gp120 binding to both CCR5 and CXCR4 was fully inhibited by 1:1 stoichiometric condition of mCD4-$P3YSO_3$. The overall positive charge of the V3 loop, which is much higher in X4- than in R5-gp120 (Moulard, Lortat-Jacob et al. 2000) strongly influences the electrostatic potential of the coreceptor binding region of the protein. In the case of CXCR4-using viruses electrostatic interactions between the sulfated peptide and the V3 loop may thus also participate in the blocking mechanism. This view is consistent with the fact that the V3 loop (which importantly contributes to coreceptor binding) is located close to the CD4i bridging sheet and with its known capacity to interact with polyanions (Moulard, Lortat-Jacob et al. 2000). This is further supported by the observation that all the anionic peptides prepared during the course of this study (mCD4-P3Asu, mCD4-P3pF, mCD4-E13 and mCD4-P3Y) also display some level of antiviral activity against X4- but not against R5- viruses. This also suggests that, in engineering such compounds, it should be advantageous to use sulfated peptides with only modest specificity so that they can broadly target distinct envelopes, the high specificity of the conjugated bivalent compound being brought by the mCD4 moiety. Structural studies of mCD4-$P3YSO_3$, in complex with different gp120 would be interesting approaches to further define these aspects. In this regard, it can be noted that sulfated peptides would represent an advantage over HS, the crystallography of which, in complex with proteins appearing to be specially challenging (Imberty, Lortat-Jacob et al. 2007).

Although relatively limited in molecular mass (5500 Da) the mCD4-$P3YSO_3$ molecule has the remarkable property to target two critical and conserved regions of gp120, and thus to simultaneously block two large protein surfaces (i.e. the CD4 and the coreceptor binding site).

In complete agreement with the biochemical data, it displays 1 nM ED_{50} anti-HIV-1 activity, for both CXCR4 and CCR5 using model viruses in a cellular assay. Importantly, we also found that this compound had a broad neutralizing activity and was very effective against a number of HIV-1 clinical isolates, strongly suggesting that this approach deserves further investigation toward *in vivo* evaluation. No effective antagonistic inhibitors yet exist for CXCR4. This compound, which at 1 μM is devoid of toxicity, could be a valuable weapon against the more aggressive CXCR4-tropic HIV-1 strains or for patients featuring a mixed HIV-1 population for which CCR5 antagonist cannot be used.

SIGNIFICANCE

While very significant progress has been made in the development of anti-HIV-1 drugs, the emergence of drug-resistant viruses, the inability of current therapy to be curative and its adverse side effects has led to an urgent need for new blocking strategies. As a target, gp120 which features the coreceptor binding site is particularly attractive. However its cryptic nature makes it a difficult target which up to now has resisted attacks.

Here we covalently linked a sulfotyrosine containing tridecapeptide that targets the gp120 coreceptor binding site, to a CD4 mimetic (mCD4). We showed that the mCD4, in interacting with gp120, induces conformational changes that expose the coreceptor binding site and renders it available to be blocked by the sulfated peptide. In cellular assays, this compound, which successfully targets two critical domains of gp120, displays strong antiviral activities and neutralizes HIV-1 with 1 nM IC_{50}.

The conjugate was much more effective than a mixture of mCD4 and tridecapeptide alone, indicating that the covalent linkage is essential to produce a synergistic effect. This compound establishes a new type of inhibitor and suggests a concept by which a relatively low specific molecule (the sulfated peptide), coupled to a highly specific compound (the mCD4) can reach very high affinities for its target. Combining these two characteristics, may enable the molecule to accommodate mutations that invariably characterize acquired viral resistance.

These results should have strong implications for the development of a new class of anti-HIV-1 therapy: the mCD4-conjugate simultaneously blocks the attachment and entry domains of gp120 and thus inhibits viral replication at a very early stage of the viral life cycle. Most importantly, it has the remarkable and unique property to neutralize both CCR5- and CXCR4-

tropic HIV-1. This is definitively a strong advantage since HIV-1 may escape from CCR5 antagonists through selection of CXCR4-using variants.

EXPERIMENTAL PROCEDURES

Materials

A BIAcore 3000 machine, CM4 sensorchip, amine coupling kit and HBS-P (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P20, pH 7.4) were from GE-Healthcare. Streptavidin and Piperidin were from Sigma. MN and YU2 gp120 were from Immunodiagnostic. Soluble CD4, mAb 17b and Cf2Th coreceptor expressing cells were obtained through the NIH AIDS Research and Reference Reagent Program. The antibodies 12G5 and 2D7 were purchased from R&D systems and BD pharmingen respectively. The HIV-1 entry inhibitors AMD3100 and Maraviroc were from Fernando Arenzana (Pasteur Institute, Paris). The1D4 antibody was from Flint Box, University of British Columbia. Synthetic phospholipid blend 1,2-dioleoyl-sn-glycero-3-phosphocholine/1,2-dioleoyl-sn-glycero-3-phospho-L-serine formulation (DOPC/DOPS; 7:3, w/w), the Mini-Extruder kit, filter supports and polycarbonate filters with defined pore diameter (100nm) were purchased from Avanti Polar Lipids. Detergents, n-dodecyl-β-D-maltopyranoside (DOM), 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane sulfonate/N,N-Dimethyl-3-sulfo-N-[3-[[3α,5β,7α,12α)-3,7,12-trihydroxy-24-oxocholan-24-yl]amino]propyl]-1-propanaminium (Chaps) and Cholesteryl hemisuccinate tris salt (CHS) were purchased from Anatrace. Complete, EDTA-free protease inhibitor tablets were from Roche Diagnostics. Polyethylene glycol 8,000 50% w/v solution was purchased from Hampton research. Resins for peptide synthesis were purchased from RAPP Polymere GmbH and Fmoc AAs, HATU, NMP, DMF, TFA were from Applied Biosystems. Fmoc-Tyr (SO3.NnBu4)-OH and Fmoc-γ-Aminobutyric-OH (γ-Abu) were from Novabiochem, (S)-Fmoc-2-amino-octanedioc acid-8-ter-butyl ester (Asu) from Polypeptides, and Fmoc -L-4 (O-tButylcarboxymethyl)-Phe-OH

(pF) from Anaspec. HPLC grade triethylamine acetate buffer was from GlenResearch. *N*-succinimidyl-S-acetylthiopropionate (SATP) was from Pierce.

CCR5/CXCR4 solubilization

The human receptors CCR5 and CXCR4, featuring a C-terminal C9 tag (TETSQVAPA), were expressed in Cf2Th canine thymocyte cells as described previously (Mirzabekov, Bannert et al. 1999). The CCR5 and CXCR4 solubilization protocol was adapted from a described procedure (Navratilova, Sodroski et al. 2005). Briefly Cf2Th.CCR5 or CXCR4 expressing cells (5-8x10^6) were solubilized in 1 ml buffer consisting of 100 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 7.5), 10% glycerol, 15% PEG 8000, protease inhibitors, CHS (0.2%), DOM (1.5%), CHAPS (1.5%) and 0.33mM DOPC:DOPS liposomes (see detailed buffer preparation in the supplemental experimental procedures). The cell suspension was sonicated (6 x 1s pulses) and placed on a rotating wheel at 4°C for 3 hours. The solutions containing the solubilized coreceptors were centrifuged at 14 000 rpm for 30 minutes at 4°C and the supernatants were either used directly in SPR analysis or stored at -80°C until further use.

Surface plasmon resonance (SPR) based binding platform

The interactions between gp120 and its ligands (CD4, mAb 17b, CCR5 and CXCR4) were analyzed by SPR technology. For that purpose, N-ethyl-N'-(diethylaminopropyl)-carbodiimide (EDC)/N-hydroxy-succimide (NHS) activated CM4 sensorchips were functionalized with either 1200 RU of soluble CD4, 700 RU of mAb 17b or 7000 RU of mAb 1D4 and blocked with pH 8.5 1M ethanolamine. The C9-tagged CCR5 or CXCR4 were captured onto the 1D4 mAb to a level of ~ 4000 RU. In some cases, gp120 were also immobilized onto CM4 sensorchip. For this, MN (50µg/ml in 5 mM maleate buffer, pH 6) or

YU2 (50µg/mL in 10 mM acetate buffer, pH 4.8) were injected at 5 µL/min over an EDC/NHS activated flow cell until levels of 4500 RU was obtained. Molecules under investigation were injected over the different surfaces and the binding responses were recorded as a function of time (see supplemental experimental procedures).

Peptide synthesis and purification

Peptides were prepared by solid-phase peptide synthesis on H-Ser(tBu)-2-ClTrt-PS-resin using Fmoc chemistry excepted for the E13 peptide which was prepared on Fmoc-Glu(tBu)-PHB-PS-resin. Fmoc-Tyr-(SO3.NnBu4)-OH was used to synthesize the sulfotyrosines containing peptide. SATP was used to introduce a protected sulfhydryl groups at the N-terminus of each purified peptide, which were then conjugated in presence of hydroxylamine to a K^5 maleimide-activated mCD4, the synthesis of which has been reported elsewhere (Baleux, Loureiro-Morais et al. 2009) to yield the desired conjugates mCD4-P3YSO_3, mCD4-P3Y, mCD4-P3pF, mCD4-P3Asu and mCD4-E13. All compounds were purified by RP-HPLC. Analytical procedures, characterization and quantification of these materials are described in the supplemental information.

Antiviral Assay

Phytohemagglutinin (PHA)-P-activated PBMCs were infected either with the reference lymphotropic HIV-1/LAI strain (Barre-Sinoussi, Chermann et al. 1983) or with the reference macrophage-tropic HIV-1/Ba-L strain (Gartner, Markovits et al. 1986). These viruses were amplified *in vitro* with PHA-P-activated blood mononuclear cells. Viral stocks (including clinical isolates) were titrated using PHA-P-activated PBMCs, and 50% tissue culture infectious doses ($TCID_{50}$) were calculated using Kärber's formula (Kärber 1931). Viruses

(125 $TCID_{50}$) were incubated for 30 min with five concentrations (1:5 dilutions between 500 nM and 320 pM) of each of the molecules to be tested and added to 150 000 PBMCs (m.o.i. ~ 0.001). Cell supernatants were collected at day 7 post-infection and stored at -20 °C. In some cases, the compounds were added to the cells prior to viral challenge. Viral replication was measured by quantifying reverse transcriptase (RT) activity in the cell culture supernatants using the Lenti RT Activity Kit (Cavisi) and AZT was used as reference anti-HIV-1 molecule. In parallel, cytotoxicity was evaluated on day 7 in uninfected PHA-P-activated PBMC using a colorimetric methyl-tetrazolium salt (MTS/PMS) assay (Promega). Experiments were performed in triplicate and 50, 70 and 90% effective doses (ED) were calculated using SoftMaxPro software.

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FIGURE LEGENDS

Figure 1: Ligand binding to CCR5 and CXCR4 immobilized sensorchips

Carboxy-terminal C9 tagged CCR5 or CXCR4 were solubilized from Cf2Th cells and captured on top of a mAb 1D4 activated CM4 sensorchip. CCR5 (left) and CXCR4 (right) ligands were injected over the coreceptor surfaces, and the binding responses (in RU) were recorded as a function of time (in S). Binding of 25 nM of mAb 2D7 (blue) and mAb 12G5 (red) to CCR5 (A) and CXCR4 (B). Binding of YU2 gp120 (black), YU2/mCD4 (blue) or YU2/mCD4/maraviroc (red) to CCR5 (C) or MN gp120 (black), MN/mCD4 (blue) or MN/mCD4/AMD3100 (red) to CXCR4 (D). Binding of the equimolar complex of YU2/mCD4 at (from top to bottom) 100, 66, 44, 29, 19 and 12.5 nM to CCR5 (E) or equimolar complex of MN/mCD4 at (from top to bottom) 225, 150, 100, 66, 44 and 29 nM to CXCR4 (F). The black traces correspond to the experimental data, and the red traces correspond to the fitted data using a 1:1 langmuir model. Binding of YU2 or MN gp120 (100 nM) preincubated with 100 nM of mCD4 (blue) or mCD4-HS_{12} (red) to CCR5 (G) or CXCR4 (H).

Figure 2: mCD4-S(XDXS)$_3$ constructs

A miniCD4 was used as a CD4 binding site (CD4BS) ligand and covalently conjugated through an appropriate linker to $S(XDXS)_3$ peptides investigated as potential coreceptor binding site (CoRBS) ligands. S and D are serine and aspartic acid residues respectively and X is either a sulfotyrosine (Y_{SO3}), a p-carboxymethyl phenylalanine (pF) an aminosuberic acid (Asu) or a tyrosine (Y).

Figure 3: The $S(XDXS)_3$ HS mimetic peptides coupled to mCD4 inhibit gp120-CD4, gp120-mAb 17b and gp120-coreceptor interactions through binding to the CD4 and the coreceptor binding sites of gp120

Binding responses measured when YU2 (A) or MN (B) gp120 at 100 nM, either alone (blue) or preincubated with 100 nM of mCD4 (pink), mCD4-P3Y (green), mCD4-E13 (turquoise), mCD4-P3pF (orange), mCD4-P3Asu (brown) or mCD4-$P3YSO_3$ (red) were injected over a CD4 activated surface. YU2-mCD4 (C) or MN-mCD4 (D) complexes (25 nM) were preincubated with 5 µM of HS_{12} (red), $P3YS0_3$ (green) or the other HS mimetic peptides (none, P3Y, E13, P3pF and P3Asu; all in black) and injected over a mAb 17b activated surface. The blue trace shows the binding of gp120 to mAb 17b in the absence of mCD4. The $P3YSO_3$ peptide (E) or HS_{12} (F) at different concentrations were coincubated with YU2-mCD4 (circle) or MN-mCD4 (square) and injected over a mAb 17b surface. The binding response (mean of triplicate experiment) recorded at the end of the injection phase was plotted versus the concentration of the inhibitors in µM. Overlay of sensorgrams showing the injection of 100 nM of mCD4 (blue), mCD4-P3Y (black) or mCD4-$P3YSO_3$ (red), from 0 to 600 seconds, over immobilized YU2 (G) or MN (H) gp120, after which 15 µg/ml of mAb 17b was injected from 600 to 900 seconds. Binding of YU2 or MN gp120 (100 nM) preincubated with 100 nM of mCD4 (blue) or mCD4-$P3YSO_3$ (red) to CCR5 (I) or CXCR4 (J). In all graphs, binding signals were recorded in RU as a function of time (S).

Figure 4: Antiviral activity of mCD4 linked to either HS_{12} or $S(XDXS)_3$ HS mimetic peptides

PHA-P-activated PBMCs were infected with either (A) LAI (X4 tropic) or (B) Ba-L (R5 tropic) HIV-1 strains, preincubated with each of the drugs under investigation (1:5 dilutions

between 500 nM and 320 pM). Molecules and viruses were maintained throughout the culture, and cell supernatants were collected at day 7 post-infection. Reverse transcriptase activity was quantified from which 50 (black), 70 (grey) and 90% (white) effective doses (ED) were calculated. In the absence of the inhibitory compounds, the RT level was in the range of 10000-25000 and 6500-10000 pg/ml (depending on the donor) for LAI and Ba-L strains respectively. Data are represented as mean of triplicate experiments (± SEM) performed on PBMCs from three to four donors.

Table 1: Anti-HIV-1 activity of AZT, mCD4-P3YSO$_3$, P3YSO$_3$ and mCD4 against clinical HIV-1 isolates

Viral strain: Clade-tropism		92UG029 A-X4	SF162 B-R5	92US723 B-R5/X4	96USHIPS4 B-R5/X4	92HT599 B-X4	98IN017 C-X4
AZT	ED_{50}	7±0	8 ±7	8±0.1	19±9	9±4	8±3
	ED_{70}	16±3	13±8	17±1	27±11	22±5	19±5
	ED_{90}	61±17	31±3	59 ±19	56±15	110±13	108±25
mCD4-P3YSO$_3$	ED_{50}	0.2±0.0	0.3±0.2	0.3±0.1	1.2±1	0.5±0.2	29±18
	ED_{70}	0.3±0.1	0.4±0.3	0.35±0.2	1.6±1.2	1.3±0.9	147±9
	ED_{90}	0.8±0.3	0.9±0.2	0.45±0.2	3±1.4	3.5±0.0	> 500
P3YSO$_3$	ED_{50}	> 500	> 500	> 500	> 500	> 500	> 500
	ED_{70}	> 500	> 500	> 500	> 500	> 500	> 500
	ED_{90}	> 500	> 500	> 500	> 500	> 500	> 500
mCD4	ED_{50}	403±76	245±155	23±1	> 500	355±155	> 500
	ED_{70}	> 500	352±105	34±10	> 500	> 500	> 500
	ED_{90}	> 500	> 500	52±22	> 500	> 500	> 500

The table shows the effective dose (ED, mean of triplicate determination), in nM (± s.d.) required to inhibit 50, 70 and 90 % of HIV-1 replication.

Figure 1

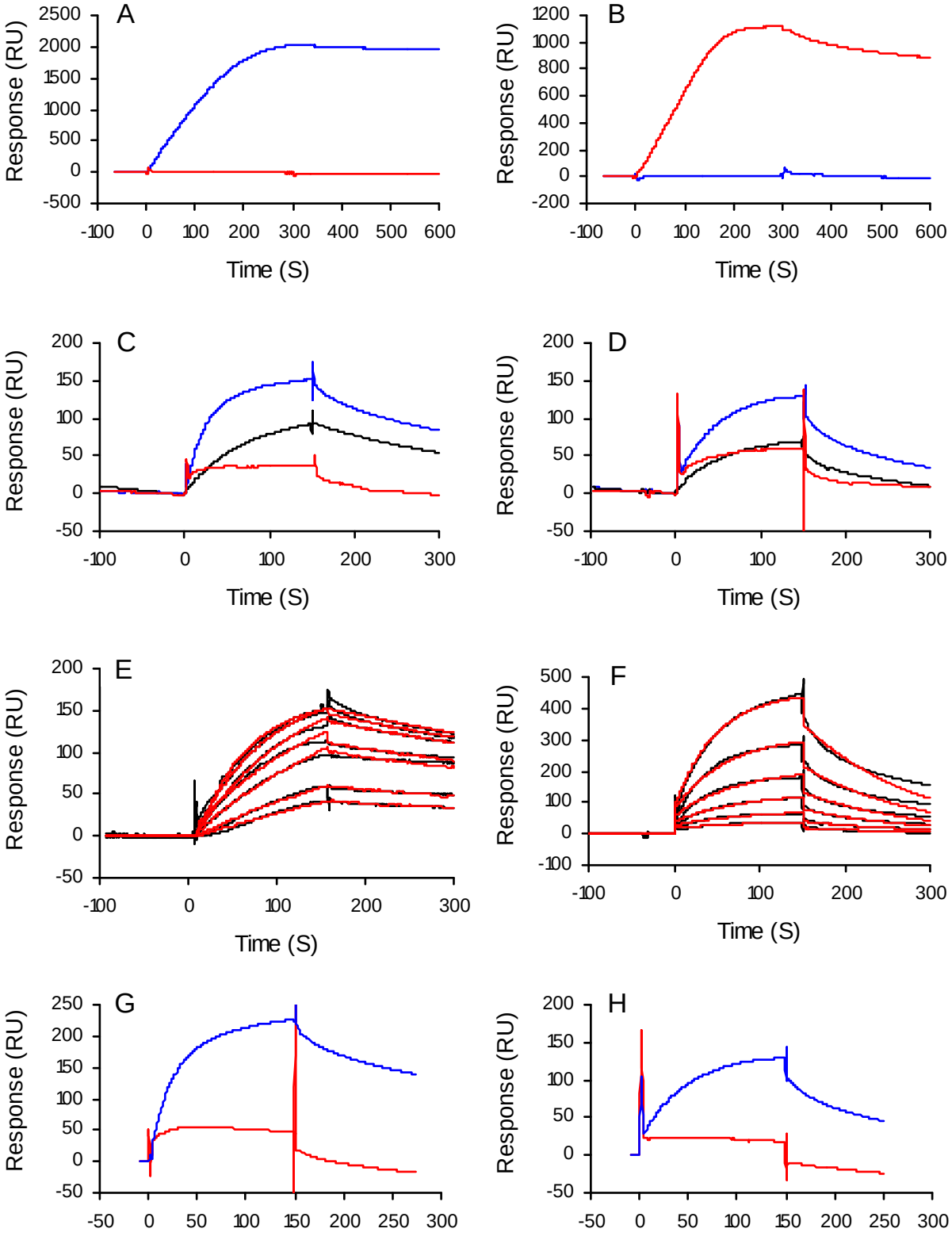
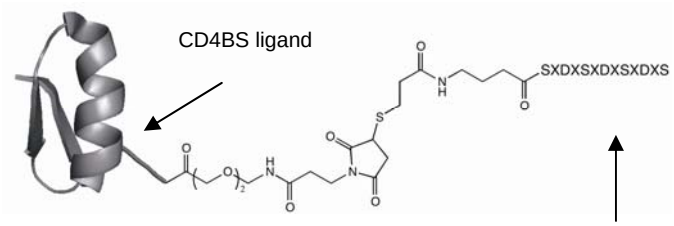
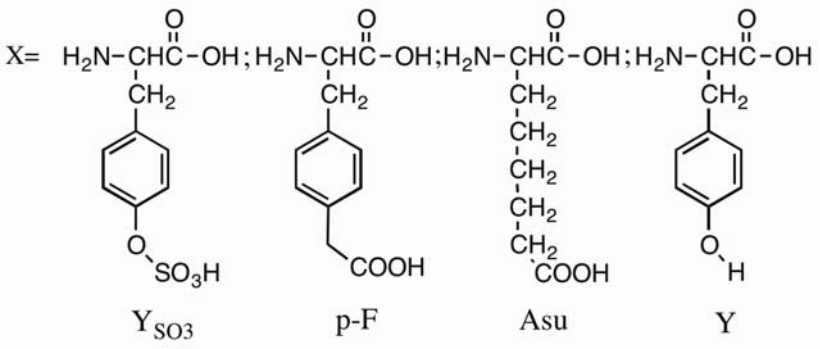


Figure 2





X	**S(XDXS)$_3$ name**
YSO$_3$	P3YSO$_3$
pF	P3pF
Asu	P3Asu
Y	P3Y

Figure 3

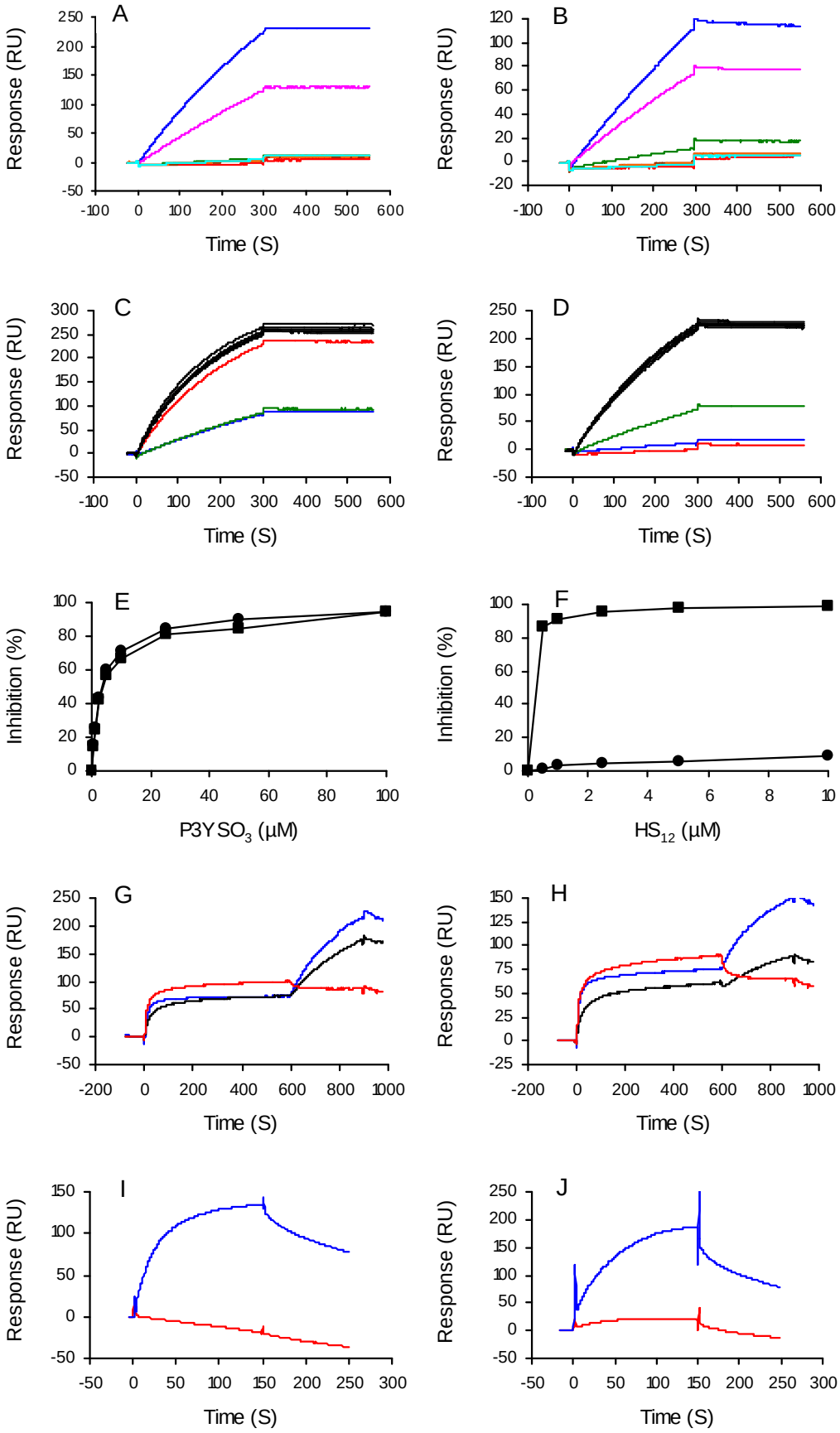
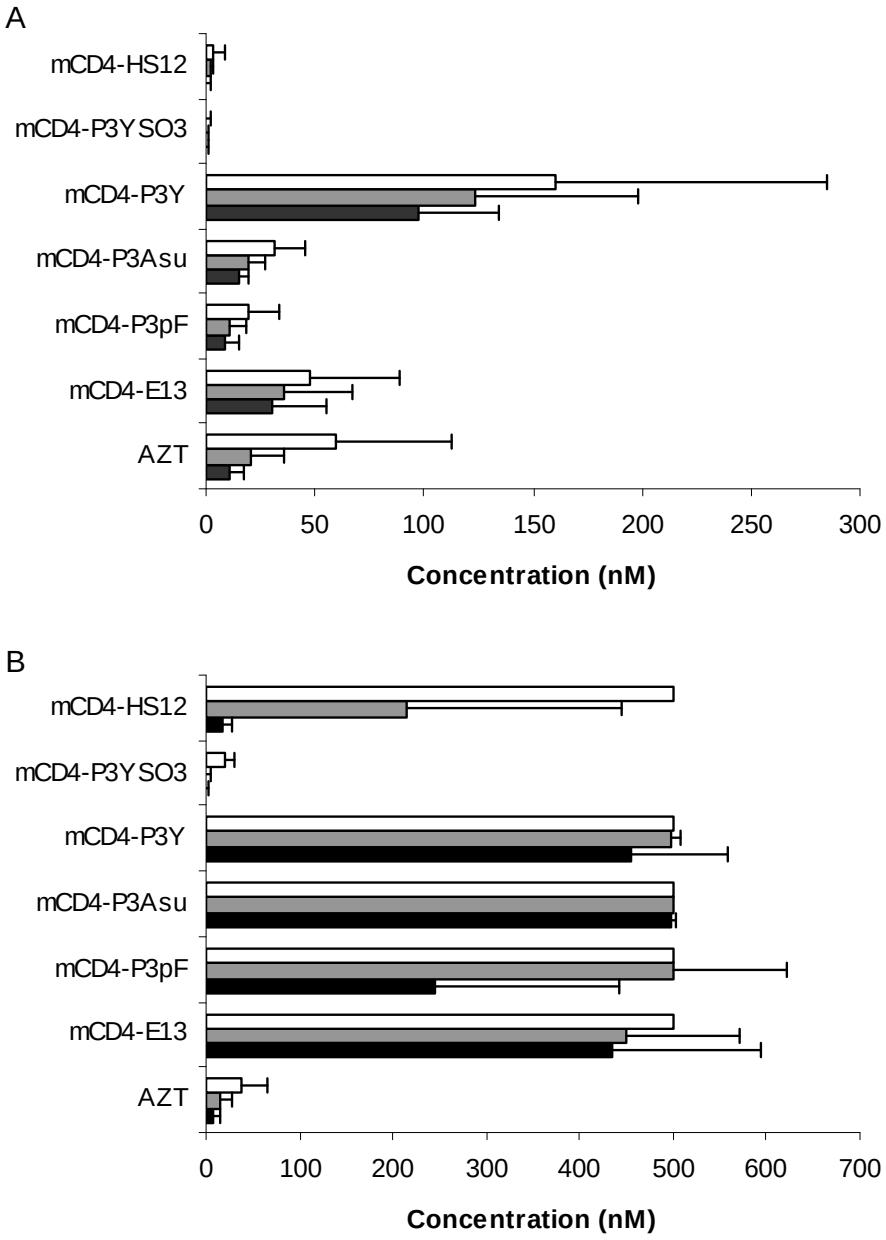

A
Response (RU)
Time (S)
B
Response (RU)
Time (S)
C
Response (RU)
Time (S)
D
Response (RU)
Time (S)
E
Inhibition (%)
P3YSO3 (µM)
F
Inhibition (%)
HS12 (µM)
G
Response (RU)
Time (S)
H
Response (RU)
Time (S)
I
Response (RU)
Time (S)
J
Response (RU)
Time (S)

Figure 4


A
mCD4-HS12
mCD4-P3YSO3
mCD4-P3Y
mCD4-P3Asu
mCD4-P3pF
mCD4-E13
AZT
0
50
100
150
200
250
300
Concentration (nM)
B
mCD4-HS12
mCD4-P3YSO3
mCD4-P3Y
mCD4-P3Asu
mCD4-P3pF
mCD4-E13
AZT
0
100
200
300
400
500
600
700
Concentration (nM)

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Supplemental Information

A heparan sulfate-mimetic peptide conjugated to a mini CD4 displays very high anti HIV-1 activity independently of coreceptor usage

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Figure S1, related to Figure 2

RP-HPLC chromatograms overlay of mCD4-P3YSO$_3$, mCD4-P3Y, mCD4-P3pF, mCD4-P3Asu and mCD4-E13.

Figure S2, related to Figure 2

MS data for mCD4-P3YSO$_3$, mCD4-P3Y, mCD4-P3pF, mCD4-P3Asu and mCD4-E13.

Table S1, related to Figure 2

Table reports the synthesis yield and the characterization data (MS and HPLC retention time) at each step of the synthesis.

Table S2, related to Figure 4

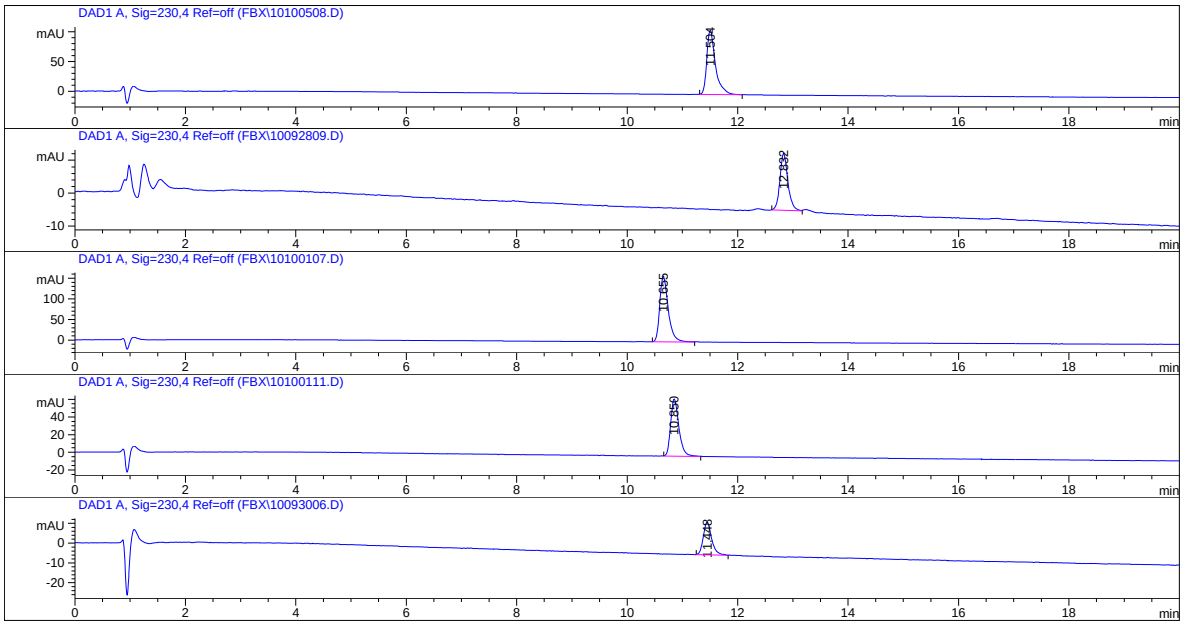
Table reports the antiviral activity of AZT, mCD4-P3YSO$_3$, P3YSO$_3$ and mCD4, measured when the molecules were either added to the virus prior to infection, or added to the cells prior to the viral challenge.

Supplemental experimental procedures, related to the:

- Peptide synthesis, conjugation, purification and characterization
- Buffer preparation for the solubilisation and capture of CCR5 and CXCR4
- Biosensor binding experiments

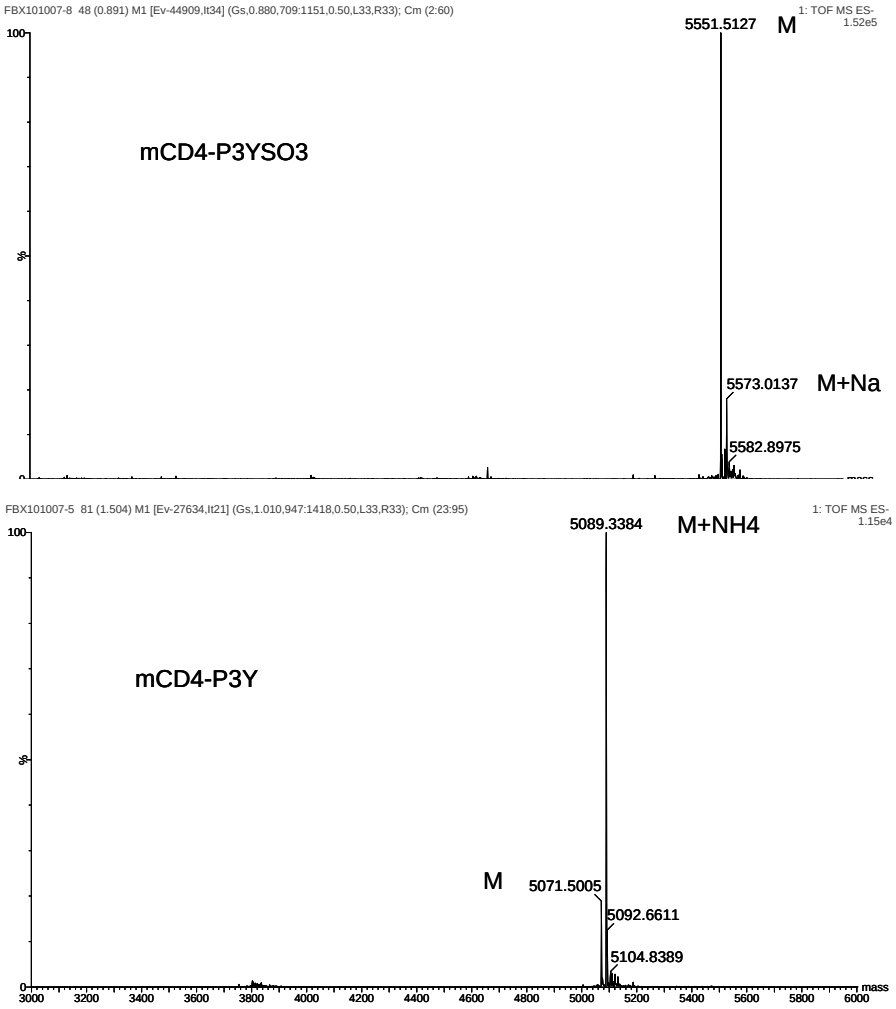
Supplemental figures

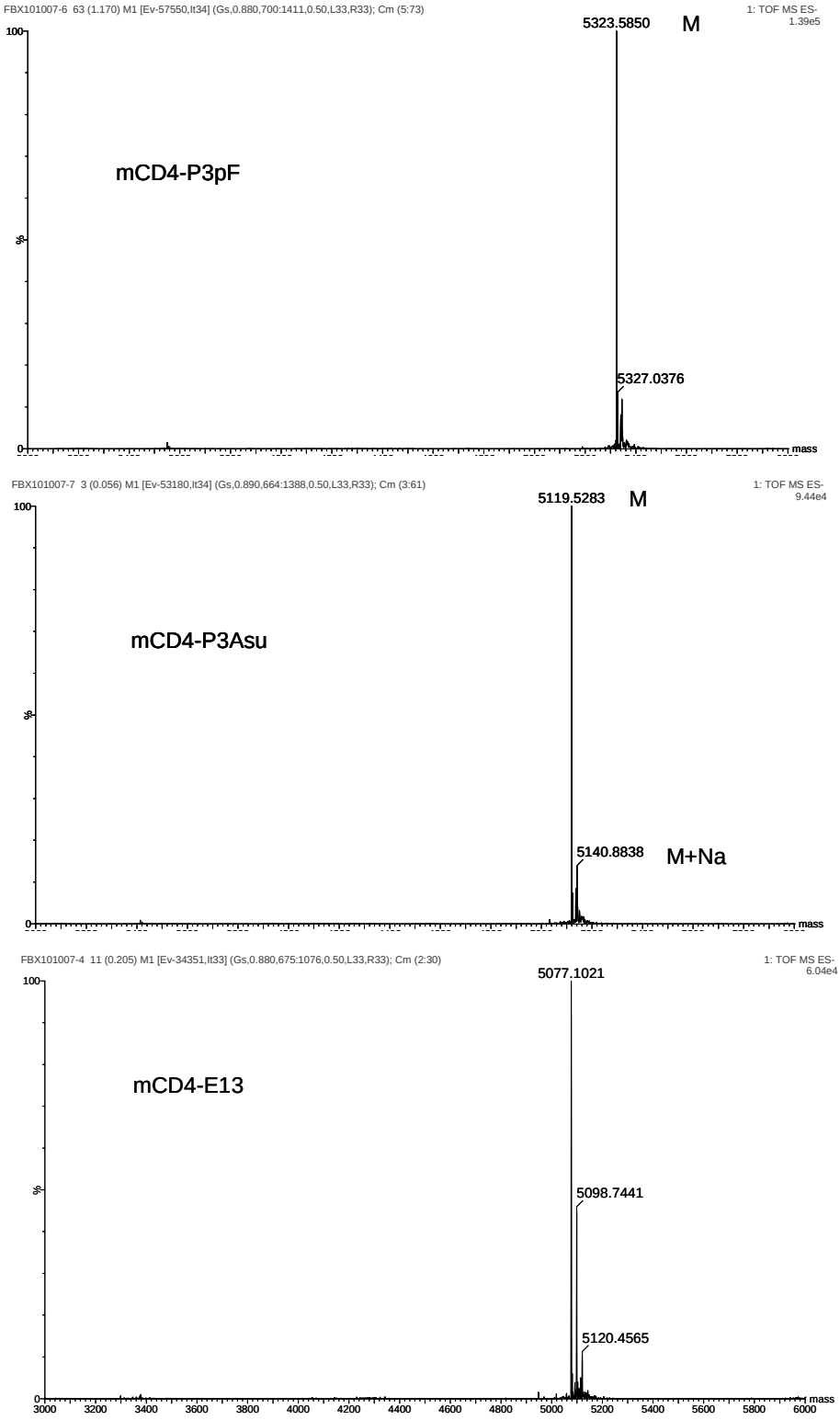
Figure S1. RP-HPLC chromatograms overlay of (from top to bottom) mCD4-P3YSO$_3$, mCD4-P3Y, mCD4-P3pF, mCD4-P3Asu and mCD4-E13.



Absorbance unit (Au) was acquired by direct injection of each conjugate in an analytical C18 RP-HPLC column, eluted with linear gradient of CH_3CN in 50 mM aqueous NEt_3-AcOH over 20 min. The traces confirmed the high purity (>95%) achieved after purification.

Figure S2. MS data for (from top to bottom) mCD4-P3YSO$_3$, mCD4-P3Y, mCD4-P3pF, mCD4-P3Asu and mCD4-E13.




FBX101007-6 63 (1.170) M1 [Ev-57550,It34] (Gs,0.880,700:1411,0.50,L33,R33); Cm (5:73)
1: TOF MS ES-
1.39e5
5323.5850
M
mCD4-P3pF
5327.0376
mass
FBX101007-7 3 (0.056) M1 [Ev-53180,It34] (Gs,0.890,664:1388,0.50,L33,R33); Cm (3:61)
1: TOF MS ES-
9.44e4
5119.5283
M
mCD4-P3Asu
5140.8838
M+Na
mass
FBX101007-4 11 (0.205) M1 [Ev-34351,It33] (Gs,0.880,675:1076,0.50,L33,R33); Cm (2:30)
1: TOF MS ES-
6.04e4
5077.1021
mCD4-E13
5098.7441
5120.4565
mass

M/z full scan spectrum was acquired by direct infusion of each conjugate in a Q-Tof Micro mass spectrometer using negative mode. Ion spray source cone voltage and collision energy were set to 5 V in order to avoid desulfation. Average mass (M), determined using MaxEnt1 software, unambiguously confirmed the identity of the compounds.

Supplemental tables

Table S1

peptide	Yield	Formula	Expected mass (monoisotopic)	Found	Retention time (min)
P3YSO$_3$	31	$C_{82}H_{98}N_{14}O_{49}S_6$	2253.3835 $[M-H]^-$	2253.3164	8.3 (10-30% over 20min)
P3Y	8	$C_{82}H_{98}N_{14}O_{31}$	1775.6601 $[M+H]^+$	1775.6370	11.7
P3pF	14	$C_{94}H_{110}N_{14}O_{37}$	2027.7235 $[M+H]^+$	2027.7490	6.2
P3Asu	10	$C_{76}H_{122}N_{14}O_{37}$	1823.8174 $[M+H]^+$	1823.8474	16.5 (0-10% over 20min)
E13	73	$C_{69}H_{100}N_{14}O_{41}$	1781.6170 $[M+H]^+$	1781.6299	12.1
Peptide-SATP	Yield	Formula	Expected mass (monoisotopic)	Found	Retention time (min) (5-25% over 20min)
P3YSO$_3$-SATP	60	$C_{87}H_{104}N_{14}O_{51}S_7$	2383.3942 $[M-H]^-$	2383.4316	11.6
P3Y-SATP	43	$C_{87}H_{104}N_{14}O_{33}S_1$	1903.6533 $[M-H]^-$	1903.6781	16.0
P3pF-SATP	50	$C_{99}H_{116}N_{14}O_{39}S_1$	2155.7167 $[M-H]^-$	2155.7869	9.3
P3Asu-SATP	36	$C_{81}H_{128}N_{14}O_{39}S_1$	1953.8262 $[M+H]^+$	1953.7822	15.2*
E13-SATP	34	$C_{74}H_{106}N_{14}O_{43}S_1$	1909.6181 $[M-H]^-$	1909.6188	6.3
Conjugate	Yield	Formula	Expected mass (average)	Found	Retention time (min) (20-40% over 20 min)
mCD4-P3YSO$_3$	47	$C_{221}H_{314}N_{54}O_{88}S_{13}$	5552.0933	5551.5127	11.5
mCD4-P3Y	38	$C_{221}H_{314}N_{54}O_{70}S_7$	5071.7081	5071.5005	12.8
mCD4-P3pF	67	$C_{233}H_{326}N_{54}O_{76}S_7$	5323.9318	5323.5850	10.7
mCD4-P3Asu	23	$C_{215}H_{338}N_{54}O_{76}S_7$	5119.8291	5119.5283	10.8
mCD4-E13	58	$C_{208}H_{316}N_{54}O_{80}S_7$	5077.5750	5077.1021	11.4

*10-30% linear gradient of CH_3CN in 0.08% aqueous TFA over 20 min.

The table reports the synthesis yield (%) and the characterization data (MS and HPLC retention time) at each step of the synthesis, i.e., the peptides, the SATP peptides and the mCD4-peptide conjugates.

Table S2: Anti-HIV-1 activity of AZT, mCD4-P3YSO$_3$, P3YSO$_3$ and mCD4 against LAI HIV-1

		Pre-treated cells	Pre-treated viruses
AZT	ED_{50}	16.5 ± 12	20 ± 12
	ED_{70}	33 ± 18	38 ± 18
	ED_{90}	96 ± 11	111 ± 40
mCD4- P3YSO$_3$	ED_{50}	0.5 ± 0.2	0.5 ± 0.3
	ED_{70}	0.6 ± 0.2	0.7 ± 0.3
	ED_{90}	1 ± 0.2	0.9 ± 0.2
P3YSO$_3$	ED_{50}	> 500	> 500
	ED_{70}	> 500	> 500
	ED_{90}	> 500	> 500
mCD4	ED_{50}	310 ± 190	406 ± 94
	ED_{70}	> 500	474 ± 27
	ED_{90}	> 500	> 500

The table shows the effective dose (ED, mean of triplicate determinations), in nM (± s.d.) required to inhibit 50, 70 and 90 % of HIV-1 replication, when the compounds were preincubated either with the cells or with the viruses.

Supplemental experimental procedures

Peptide synthesis and purification. Peptides P3YSO$_3$, P3Y, P3pF and P3Asu were synthesized on H-Ser(tBu)-2-ClTrt-PS-resin (100 μmoles; 0.78 mmole/g), and E13 on Fmoc-Glu(tBu)-PHB-PS-resin (100 μmoles; 0.61 mmole/g), using an Applied 433 peptide synthesizer. Chain elongation was performed using 10 equivalents of Fmoc amino acids and HATU/DIEA activation. Peptides were released from the resin by TFA/TIS/H_2O (95/2,5/2,5) treatment for 1h30 at room temperature, except for the sulfated peptide which was released at 4°C (ice bath). The crude peptides were isolated by cold diethyl ether precipitation, solubilised in water by adding 3% NH_4OH, except the sulfated peptide that was rapidly dissolved in 100 mM ammonium hydrogen carbonate buffer. After lyophilisation, the crude peptides were purified by C18 RP-HPLC using 50 mM aqueous NEt_3-AcOH (100 mM for the sulfated and E13 peptides) and CH_3CN as eluents. Purified peptides were analysed by mass spectrometry (Waters ionspray Q-TOF–micro) and quantified by amino acid analyses (Hitachi L-8800 apparatus). Peptide purity was controlled by analytical C18 RP-HPLC using a linear gradient of CH_3CN in 50 mM aqueous NEt_3-AcOH over 20 min (Waters Symetry C18-300Å, 3.5 μm, 2.1x100 mm column, 0.35 ml/min flow rate). See Table S1.

S-acetylthiopropionate peptides. For the S-acetylthiopropionate peptides preparation, peptides were dissolved in 50 mM sodium phosphate buffer pH 8 (1 mM final concentration). The S-acetylthiopropionate group was introduced via stepwise addition of 10 equivalents of *N*-succinimidyl-S-acetylthiopropionate (SATP; 0.26 M in DMSO) over a 40 min period. After 1h30, S-acetylthiopropionate peptides were purified by C18 RP-HPLC using linear gradient of CH_3CN in 50 mM aqueous NEt3-AcOH over 20 min (C18-300Å, 5 μm, 10x250 mm column, 6 ml/min flow rate). The SATP derived peptides purity was controlled by analytical C18 RP-HPLC using linear gradient of CH_3CN in 50 mM aqueous NEt_3-AcOH over 20 min (Waters Symetry C18-300Å, 3.5 μm, 2.1x100 mm column, 0.35 ml/min flow rate). See Table S1.

mCD4-peptide conjugates. Maleimide activated miniCD4 (mCD4-Mal) was prepared as described in WO/2009/098147, WO/2008/015273 and reference Baleux et al. 2009 of the

main manuscript. For peptide coupling to mCD4-Mal, SATP peptides were dissolved in 100 mM sodium phosphate buffer pH 7.2 (1 mM final concentration), after which 100 µl of 0.5 M NH_2OH, HCl in 100 mM sodium phosphate buffer (pH adjusted to 7.2 by 4N NaOH) was added. Deprotection of the thiol function was monitored by HPLC. After 30 min, 0.3 equivalent of mCD4-Mal in H_2O (1.5 mM) was added. After another 30 min, mCD4-peptide conjugates were purified by C18 RP-HPLC using a linear gradient of CH_3CN in 50 mM aqueous NEt3-AcOH over 20 min (C18-300Å, 5 µm, 10x250 mm column, 6 ml/min flow rate). mCD4-peptide conjugates were controlled by analytical C18 RP-HPLC using linear gradient of CH_3CN in 50 mM aqueous NEt_3-AcOH over 20 min (Waters Symetry C18-300Å, 3.5 µm, 2.1x100 mm column, 0.35 ml/min flow rate), negative mode mass spectrometry and quantified by amino acid analysis. See Figure S1, S2 and table S1.

Liposome and buffer preparation for CCR5/CXCR4 solubilisation. To prepare liposomes (final concentration of 3.3 mM), a pre-determined volume of synthetic Phospholipid Blend DOPC:DOPS (7:3, w/w) was transferred into a glass test tube and a thin lipid film was formed on the side walls of the glass tube glass by rotating the tube while evaporating all the chloroform using a stream of nitrogen gas. Once all the chloroform had been evaporated, a HEPES buffer (50mM Hepes, 150 mM NaCl, pH 7.0) was added to dissolve the dry lipid films. The lipid mixture was vortexed to facilitate dissolving the films, then the mixture was frozen, thawed, and vortexed four times. Unilamellar vesicles (ULC)/liposomes were prepared by classical extrusion through a 100 nm pore diameter polycarbonate filter using an Avanti Mini-Extruder kit. Liposomes were freshly extruded for every experiment. The 0.33mM liposome preparation was then mixed into a solution of 100 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 7.5), 10% glycerol, 15% PEG 8000, CHS (0.2%), DOM (1.5%), CHAPS (1.5%) supplemented with protease inhibitors (EDTA free Complete from Roche) and used for CCR5 and CXCR4 solubilisation.

Preparation of the Biacore binding surfaces. For sensorchip immobilization of CCR5 and CXCR4, the 1D4 antibody (recognizing the coreceptor C9 C-terminal tag) was first cross linked to a level of 7000 RU onto a CM4 sensorchip. This was performed by activation of the chip surface with 50 µL of 0.2 M N-ethyl-N'-(diethylaminopropyl)-carbodiimide (EDC) and 0.05 M N-hydroxy-succimide (NHS) at 5 µL/min followed by a 12 minutes injection of 1D4

at 100 μg/ml in 10 mM sodium acetate buffer, pH 4.2 and a 5 mins injection of 1M ethanolamine. The 1D4 surface was then equilibrated into a running buffer consisting of 50mM HEPES pH 7.0, 150 mM NaCl, 5% glycerol, 5 % PEG 8000, 5 μM $CaCl_2$, 1 μM $MgCl_2$, 0.1 % DDM, 0.1 % CHAPS, 0.02 % CHS, 5 μM of 7:3 DOPC:DOPS and 0.1 mg/ml BSA. Solubilized CXCR4 or CCR5, in the above described liposome preparation, were captured via the interaction between its C9 tag and the 1D4 mAb to a level of approximately 3000-5000 RU. The system was then equilibrated with the running buffer at 5 μl/min for approximately 20 minutes.

Other binding surfaces were prepared by injecting over EDC/NHS activated CM4 sensorchips CD4 (10 μg/mL in 10 mM acetate buffer, pH 5), streptavidin (200 μg/mL in 10 mM acetate buffer, pH 4.2), mAb 17b (5 μg/mL in 10 mM acetate buffer, pH 5), MN gp120 (50 μg/ml in 5 mM maleate buffer, pH 6) or YU2 gp120 (50 μg/mL in 10 mM acetate buffer, pH 4.8). This was performed at 5 μL/min until levels of 1200 (for CD4), 700 (for mAb 17b), 3000 (for streptavidin) or 4500 (for gp120s) RU were achieved. Surfaces were then blocked with pH 8.5 1 M ethanolamine during 5 minutes.

Biosensor binding experiments and sensorgram evaluation. Samples under investigation were prepared in HBS-P running buffer when injected at 10 μl/ml over CD4, mAb 17b or 5 μl/mL over the gp120 surfaces. Streptavidin was used as a reference surface, and binding signals were recorded with on line subtraction of control sensorgrams. Surfaces were regenerated by 1 min injection of 10 mM HCl. For binding studies on immobilized CCR5 or CXCR4, samples under investigation were prepared in 50mM HEPES pH 7.0 buffer, 150 mM NaCl, 5% glycerol, 5 % PEG 8000, 5 μM $CaCl_2$, 1 μM $MgCl_2$, 0.1 % DDM, 0.1 % CHAPS, 0.02 % CHS, 5 μM 7:3 DOPC:DOPS and 0.1 mg/ml BSA (running buffer). 1D4 was used as a reference surface. After each binding cycle, performed at 30 μl/ml, the 1D4 surface was regenerated with 10 mM NaOH containing 1% n-octyl-β-D-glucopyranoside at 100 μl/min, and subsequently reloaded with either CCR5 or CXCR4. Alternatively, to avoid this regeneration step, the gp120-coreceptor complex was washed with running buffer, until the signal returned to the baseline level (usually 30-60 min).

The binding curves obtained when gp120-CD4 complexes were injected over either CCR5 or CXCR4, were evaluated with the Biaevaluation 3.1 software. Data were analyzed by fitting of both association and dissociation phases for several concentrations, using a simple 1:1 binding

model. Several parameters (including the conformational flexibility of gp120 and the reversible nature of the gp120-mCD4 complex) were likely to complicate the binding kinetics and the values reported should be considered as estimates only. The affinities (dissociation equilibrium constants: K_d) were calculated from the ratio of dissociation and association rate constants ($K_d = k_{off}/k_{on}$).

6.5 Discussion

Currently, there is no effective anti-HIV-1 vaccine and there is no entry inhibitor that is capable of inhibiting both X4 and R5 HIV-1 viral strains simultaneously at the level of the gp120 – coreceptor interaction. Maraviroc is the first and only CCR5 antagonist which has been approved for treatment in HIV-infected patients. Maraviroc binds to a small hydrophobic pocket inbetween the transmembrane helices and thus induces a slight conformational change in the coreceptors which renders it non-recognizable by HIV-1 (Dorr, Westby et al. 2005). However, as with most antiretrovirals, the high replication rate and mutation rate of HIV-1 permits it to eventually develop resistance to Maraviroc and the envelope adapts in such a way that it is able to recognize the drug-bound confirmation of the CCR5 coreceptor. Another short-coming of the use of an entry inhibitor that blocks solely the R5 HIV-1 strain, is the outgrowth of CXCR4-tropic, more virulent HIV-1 isolates that were present at low frequencies prior to the initiation of therapy or new infections of X4 HIV-1. Thus, it is very important to target both R5 and X4 viruses simultaneously.

In the early stages of HIV-1 infection (via sexual transmission), it is believed that heparan sulphate (HS) aids to concentrate the virus on the mucosa (Saidi, Magri et al. 2007), thus bringing it into close contact with its host cell receptors, CD4 and CCR5. HS has also been shown to play a possible role in transporting HIV-1 through the blood-brain barrier during the late stages of AIDS (Argyris, Acheampong et al. 2003). Also, cells that are treated with HS degrading enzymes, demonstrated a reduced HIV-1 attachment and infection, thus these anionic polysaccharide molecules were believed to play an important role in HIV-1 infection (Ohshiro, Murakami et al. 1996; Mondor, Ugolini et al. 1998; Saphire, Bobardt et al. 2001). The interaction between HIV-1 gp120 and HS has mainly been attributed to the V3 loop, however heparin binding domains have also been identified in the V2 loop, in the C-terminal domain and within the CD4 induced bridging sheet (Crublet, Andrieu et al. 2008). A bivalent entry inhibitor (mCD4-HS_{12}) has been developed which consists of a mini CD4 molecule (mCD4) which is covalently attached to a highly sulphated 12mer oligosaccharide (HS_{12}, contains 18 sulphate residues). This molecule initially binds the CD4-binding site (CD4BS) on gp120 with the mCD4 moiety, induces the conformational changes necessary to expose the coreceptor binding site (CoRBS), and due to the small size of the HS_{12} (3 kDa, 50 times smaller than a neutralizing antibody), it is able to rapidly and effectively bind to the CoRBS and block entry through both CCR5 and CXCR4 pathways, resulting in nanomolar antiviral activity (Baleux, Loureiro-Morais et al. 2009).

Heparan sulphates are exceedingly complex molecules and unlike DNA and proteins, they do not have a blue print template or coding sequence. The basic unit is a disaccharide which consists of a uronic acid (either α-L-iduronic acid [IdoA] or β–D-glucuronic acid [GlcA]) linked (1→4) to a D-Glucosamine (GlcN). Specialised enzymes (N-deacetylase/N-sulphotransferase, epimerase, and *O*-sulphotransferase) act in an organised and regulated fashion to sculpt the mature, sulphated polysaccharide chains. Not all residues in the disaccharide are modified, thus there is an enormous level of variation in an HS oligosaccharide chain. For

example, a DNA sequence of 6 base pairs can generate 4^6 or 4096 possible different sequences. For a hexapeptide, we can have 20^6 or 64 million different possibilities (Shriver, Liu et al. 2002). However for a 12mer of HS (six disaccharide units), there can be 48^6 (12 billion) different possible sequences, which is a staggering degree of variation. The regulation of the HS biosynthetic process is poorly understood, however extremely important for a myriad of biological processes and pathological states. Enormous structural complexity and heterogeneity is thus generated and all these different structures have different functions in the biological system. Molecular characterization of GAGs and of their interacting partners is still in the early stages of development; they are not easily sequenced and there is a lack of routine biosynthetic and analytical tools for GAGs. Thus a technique whereby one can easily characterise the structures that resemble those of GAGs interacting with proteins, will greatly advance our understanding of how their structures relate to different functions.

In order to further develop the mCD4-HS_{12} molecule which is fully sulphated and which took one year to synthesize, we wanted to determine the minimal sulphate residues required for CoRBS binding as well as find a molecule that is faster to synthesize/purify from natural sources. One approach would have been to screen large libraries of differently sulphated HS_{12}mers. However, this would be virtually impossible due to the enormous amount of potential variations as well as the fact that obtaining reasonable quantities of pure homogeneous HS_{12} oligosaccharides is difficult if not impossible without using synthetic techniques. Finally, if a highly anti-viral HS_{12} was found, structure-function analysis would be very complicated due to the limiting techniques for oligosaccharide structural analysis. During this work, a relatively small library (12 populations) of HS_{12}mers was generated through several ion exchange chromatography runs. Each member of the HS_{12} fractionated library was screened for its ability to inhibit gp120-CD4 complexes from binding to 17b on the biacore. Finally, 50µM of the most sulphated fraction from the natural fractionated HS_{12} library was 30 times less efficient than 10µM of the HS_{12} un-fractionated mixture and 30 times less efficient than 5µM of the synthetic HS_{12}. The active HS_{12} molecule might have been 'lost' during the fractionation process which could indicate that there exists a highly active molecule in the mixture which was not identified. Therefore a different approach was necessitated to improve the specificity, affinity and speed of production of the glyco-moiety of mCD4-HS_{12}.

It is known that R5 HIV-1 relies heavily on the N-terminal and second extracellular loop of CCR5 for entry. Interestingly, the amino terminus contains several sulphated tyrosines as well as certain neutralizing antibodies (E51 and 412d) which both interact with the conserved CCR5 binding site (Farzan, Choe et al. 1998; Farzan, Babcock et al. 2002; Choe, Li et al. 2003; Huang, Lam et al. 2007). Numerous studies have exploited the properties of anionic polyanions, sulphated molecules and peptides derived from the N-terminal of chemokine coreceptors as potential inhibitors of the 17b mAb binding to the CD4i with µM range IC_{50s} (Cormier, Persuh et al. 2000; Farzan, Vasilieva et al. 2000; Cohen, Forzan et al. 2008; Crublet, Andrieu et al. 2008; Brower, Schon et al. 2009; Dervillez, Klaukien et al. 2010; Acharya, Dogo-Isonagie et al. 2011; Kwong, Dorfman et al. 2011). These studies show inhibition of 17b mAb binding to the

CD4i, however, this antibody is only a partial coreceptor surrogate as it only recognises the bridging sheet and not the V3 loop (Dragic 2001), which is another critical region involved in coreceptor binding. Thus this antibody is a poor coreceptor mimic. However, due to the difficulty in manipulating coreceptors, this mAb was used as a surrogate coreceptor.

The present study aimed to replace the mAb 17b as an 'incomplete' coreceptor surrogate and use actual coreceptors in experiments where potential entry inhibitors could be screened for their ability to bind the CoRBS. To do this, we captured either coreceptor (CCR5 or CXCR4) on the biacore surface so that HIV-1 entry inhibiting molecules could be screened for their ability to inhibit gp120-CD4 complexes from binding to their respective coreceptors. Here, we demonstrated two challenging feats in biology; firstly, we have solubilized and immobilized both CCR5 and CXCR4 in a lipid/detergent environment preserving their functional structures and for the first time, kinetic data has been determined for gp120-CD4 complexes binding to CXCR4 using surface plasmon resonance. We report a K_D of 154 ± 68 nM for X4 gp120-CD4 interaction with CXCR4 and K_D of 11.5 ± 2.9 nM for R5 gp120-CD4 interacting with CCR5. These affinities compare well with those calculated for coreceptors that remain in their natural membrane environment (Doranz, Orsini et al. 1999; Babcock, Mirzabekov et al. 2001). This assay has many advantages; most importantly, it allows the isolation of native GPCRs for specific interaction analysis, which is much a more relevant and complete approach for HIV-1 entry inhibitor screening assays as compared to using a coreceptor surrogate (e.g. mAb 17b). The assay is performed in real-time, there is no labelling required of either the ligand or the receptors, the coreceptors on the surface are re-usable after a long dissociation period and the results are reproducible. It is also very user-friendly to be able to store the pre-solubilized coreceptors at -80°C as this allows for rapid preparation time for the SPR experiments and the same 'batch' of purified coreceptors can be used for several different experiments on different days – allowing for standardisation of the results. This assay can be used for a multitude of tests to elucidate many unanswered questions on the coreceptor binding site of gp120. E.g. one can probe the V3 loop of a pre-triggered gp120 with various antibodies in search of new broadly neutralizing antibodies. However, the disadvantages of this technique are the fact that one is required to work with lipid/detergent mixtures which are complex to manipulate as they can form larger micelles and their density in a buffer can change over time which can affect binding results. Also, this technique requires long dissociation periods (which can reach up to 2 hours each) if the same coreceptor surface is required for multiple injections.

The second challenging feat which was achieved during this work was that GAG-mimetic peptides have been produced which contain sulphated amino acids that mimic the sulphated residues in the disaccharide building block of oligosaccharide chains and these peptides can be used as tools to define the number and placement of sulphated residues that are critical for a certain protein-GAG interactions. Since, there are few techniques that allow the study of structural characterisation and structure-function relationships for GAGs, the use of GAG-mimetic peptides can greatly advance this field.

Five peptides that mimic GAGs were used in this study; the $S(XDXS)_3$ sequence was used to replace the synthetic HS_{12}mer. Thus 13 amino acids residues were the equivalent length of a 12mer oligosaccharide. The sulphate, carboxyl and hydroxyl groups on a HS disaccharide were mimicked by the use of serine (S) and aspartate (D) amino acids in the $S(XDXS)_3$ sequence. Since we were interested in mimicking the SO_3 group in the GAG chains, several functional groups were placed in the $S(XDXS)_3$ sequence in the X position. Either sulphated tyrosines was used ($P3YSO_3$), or non-sulphated tyrosines (P3Y), or a p-carboxymethyl phenylalanine (P3pF), or a aminosuberic acid (P3Asu), or a charged carboxyl chain of 13 glutamic acids (E13) was also tested to see if a non-specific anionic polyanion showed an effect. Despite having set up a system where solubilized coreceptors could be used instead of mAb 17b to test coreceptor binding, we did test the peptide GAG mimics for their ability to prevent gp120-CD4 complexes from binding to a 17b surface so as to compare the peptides to existing entry inhibitory molecules. The peptide containing 6 sulphotyrosine residues ($P3YSO_3$) displayed the lowest IC_{50} of 3μM, which compares very well to other inhibitors. Interestingly, this was demonstrated with the peptide alone, it was not yet covalently bound to the mCD4 moiety.

The importance of the tyrosine sulphation in the N-terminus of CCR5 has been well documented for the binding of R5 HIV-1 variants, surprisingly, the existence of sulphated tyrosines (at positions 7, 12 and 21) on the HIV-1 coreceptor CXCR4 (Farzan, Mirzabekov et al. 1999) are not as critical for the binding and entry of X4 HIV-1 variants (Farzan, Babcock et al. 2002). Interestingly, our results show that the peptide containing 6 sulphated tyrosines, when conjugated to the mCD4 moiety (mCD4-$P3YSO_3$), displayed equivalent potent inhibition for the R5 gp120-CD4 complexes binding to solubilized CCR5 as it did for X4 gp120-CD4 complexes binding to solubilized CXCR4. Thus, perhaps the presence of the mCD4, brings the sulphated peptide so close to the CoRBS and the basic V3 loop of X4 gp120, that it is able to form electrostatic interactions and salt bridges with the CoRBS of X4 gp120. This is also true for the R5 gp120. Despite the general difference in overall V3 loop charge between X4 gp120 and R5 gp120, such an inhibitor targets both envelope tropisms.

Interestingly, interactions are made between the CoRBS and the inhibitors' sulphotyrosines, however, similar interactions are probably not observed / made when the CD4 bound envelope binds to the N-terminal of CXCR4 containing sulphotyrosines. This however, would need to be confirmed by crystallographic studies of the gp120-CD4 complex together with the mCD4- $P3YSO_3$ inhibitor. Owing to the mCD4, the affinity of the 'triggered' envelope for the linked-sulphated peptide is greatly increased and due to its small size, the spatial proximity of the sulphated peptide is so high it binds to the CoRBS strongly. Thus, a range of differently charged V3 loops (R5 variant possessing a net V3 loop positive charge of ≤ 5 and that of an X4 variant is typically higher, ≥ 5) can be targeted by the linked-GAG mimicking peptide. The sulphated inhibitory molecule should contain at least one sulphated tyrosine so that it can inhibit R5 viruses [as shown by (Huang, Lam et al. 2007), one sulphotyrosine binds at the base of the R5 V3 loop as seen in the crystal structure]. Then for the sulphated inhibitor molecule to also target X4 envelopes, it can contain more sulphotyrosine

residues because a typical X4 V3 loop has a net higher positive charge compared to that of the R5 V3 loop. Therefore, for the reason mentioned above, the inhibitory peptide must contain ≥ 1 sulphotyrosine, and preferably more sulphotyrosines so that it can target R5 envelopes and a range of X4 V3 loops, including highly basic V3 loops.

All the mCD4-linked peptide GAG mimetic inhibitors were tested in a PBMC infection assay by our collaborators at the CEA, where laboratory-adapted HIV-1 strains (HIV-1 LAI [X4] and HIV-1 Ba-L [R5]) were used to infect donor PBMCs either in the presence or absence of each inhibitor. Interestingly, when cells were infected with the X4 HIV-1 strain, all the bivalent inhibitors (except mCD4-P3Y) displayed ED_{50s} that were below that of AZT, a nucleoside analog reverse-transcriptase inhibitor that was approved for treatment of HIV-1 in 1987. However, when the cells were infected with the R5 strain, all the bivalent inhibiters were ineffective (except mCD4-P3YSO_3) at inhibiting HIV-1 entry. Again, this suggests that these anionic compounds bind stronger to the CoRBS of X4 envelopes than to the less positively charged CCR5 envelopes due to the overall higher charge carried by the V3 loop of X4 envelopes (Moulard, Lortat-Jacob et al. 2000). Thus, for both strains of HIV-1 (LAI and Ba-L), an ED_{50} as low a 1 nM was necessary for HIV-1 entry inhibition by mCD4-P3YSO_3. This is currently, the only entry inhibitor that targets both CCR5 and CXCR4 utilizing HIV-1 strains with such a low effective dose. In addition, up to 1 μM concentration, mCD4-P3YSO_3 shows no sign of toxicity towards the cell. This molecule has the potential to be used as a prophylactic prevention strategy or as a treatment for people already infected with HIV-1 (microbicide). This new bivalent molecule is relatively rapid to produce and the usage of chiral amino acids can be debated to escape recognition by host proteases and the use of sulphonate (instead of sulphate) could be considered which are more stable than sulphates.

Not only is the sulphated GAG mimetic peptide a huge success for HIV-1 entry inhibition, but this mimetic will greatly advance the glycobiology field. This is so since structural characterisation of GAGs is so cumbersome, tedious and technically challenging that the use of a peptide, where the position and type of negative change can be easily and rapidly modified, will enormously aid structure-funtion analysis. For example, with the $S(XDXS)_n$ sequence, the sulphates can be placed at different positions (on one extremity $S[X_SDX_SSXDXSXDXS]$, in the middle $S[XDXSX_SDX_SSXDXS]$ or throughout $S[X_SDX_SSX_SDX_SSX_SDX_SS]$) and this can give a more refined idea of where and which sulphated residues are critical for a certain protein-GAG interaction.

Chapter 7: Side-Projects

7.1 Screening of small natural molecules for HIV-1 entry inhibitory capacity

Our ability to analyse molecules for their ability to block gp120-HS and gp120-17b interactions, has attracted the attention of a company. In that context, using the screening system described in Figure 6.2, we investigated three naturally derived small anionic molecules, extracted from natural sources, for their ability to inhibit gp120 (either CCR5 utilizing [R5] or CXCR4 utilizing [X4]) from binding to heparan sulphate, CD4 or to mAb 17b. These molecules behave strongly as HS-like molecules, i.e. they inhibit the binding of envelopes to both HS and (in the presence of soluble CD4) mAb17b. This strongly suggests that these compounds bind to clusters of basic residues on gp120, which includes the CD4 induced epitope (coreceptor binding site) (Vives, Imberty et al. 2005) for R5 and X4 envelopes and the V3 and V2 loops (Crublet, Andrieu et al. 2008), at least for X4 envelopes. Further details on these experiments are withheld for confidentiality reasons, however, micro molar IC_{50}s were calculated for these molecules with one molecule in particular displaying a higher affinity compared to the other two. This work is currently being prepared for publication.

7.2 Molecular mechanisms underlying the increase in resistance to chemokines of R5 viruses in HIV infection

CCR5-utilizing viruses (R5) are predominant during the chronic, asymptomatic stages of HIV-1 infection, while CXCR4-utilizing viruses (X4 or R5/X4) emerge after several years later in about half of the infected individuals who progress to AIDS. Therefore, the other half of the infected individuals who progress to AIDS, develop the disease in the presence of only R5 viruses. Since CCR5 and CXCR4 are GPCRs, their natural binding partners, chemokines, are able to inhibit viral entry by one of two ways; either by sterically inhibiting gp120 from accessing the coreceptor binding site or by causing endocytosis of the coreceptor. However, according to the literature, some R5 viruses develop increased infectivity and resistance to inhibition by the chemokines during infection. To define the molecular mechanisms whereby these viruses become resistant to chemokine inhibition, our collaborators at the Institut Pasteur (Bernard Lagane) have cloned various envelopes from a longitudinal drug-naive cohort, followed-up over several years. These envelopes will be evaluated with an aim to test the hypothesis that certain changes in the R5 *env* sequence are associated with the virus's efficiency to enter host cells and resistance to CCR5 chemokine inhibition. They speculate that the resistance of certain R5 viruses to inhibition by CCR5 chemokines may be related to changes in the virus's gp120 binding affinity for CD4 and CCR5. In order to assess these assumptions, our collaborators will perform molecular pharmacology and virology experiments to characterize the receptor binding properties of purified and radioactive monomeric gp120 and they will study the

ability of different CCR5 chemokines to prevent gp120 binding to CCR5 and viral entry.

My role in this project has been to asses the binding affinity of various cloned R5 and X4 envelopes for different binding receptors using SPR (as mentioned in Chapter 6), including CD4, HS and 17b. For some of the envelopes, I have also tested their ability to bind the solubilized coreceptors. Interestingly, the affinity data I have obtained thus far for certain R5 envelopes and full length CD4, corresponds to those K_D determined by our collaborators using a competition experiment on HEK CD4 expressing cells. The binding data I obtained between the CD4-bound envelopes and 17b, also correlates with the tendency of certain envelopes to bind better to CD4 than others. Preliminary results from my experiments and those of our collaborators indicate that affinity of viral envelopes for CD4 does not increase in the course of HIV infection. This suggests that increasing viral fitness and resistance to chemokine inhibition as the disease progresses could rely on changes of other steps of the virus entry process including binding to CCR5 or HS. In particular, we propose that later-stage R5 envelopes might bind the CCR5 receptor differently with probably a higher affinity and are thus more virulent. This project will provide clues on some aspects of HIV physiopathogenesis, including the mechanisms by which phenotypic properties of R5-viruses evolve during the course of infection and contribute to disease progression. It will shed light on the mechanisms that account for the enhanced ability of R5 viruses to infect cells expressing low levels of HIV-1 entry receptors in late stages of infection (*i.e.* monocytes, MDMs and perhaps central memory $CD4^+$ T lymphocytes).

Chapter 8: Methods

8.1 Materials

8.1.1 Biacore reagents and antibodies

A BIAcore 3000 machine, CM4 sensorchip, amine coupling kit and HBS-P (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P20, pH 7.4) were from GE-Healthcare. Streptavidin was from Sigma. MN and YU2 gp120 were from Immunodiagnostic. Soluble CD4, mAb17b and Cf2Th coreceptor expressing cells were obtained through the NIH AIDS Research and Reference Reagent Program from Dr. Tajib Mirzabekov and Dr. Joseph Sodroski. The antibodies 12G5, 12G5-conjugated to Fluorescein isothiocyanate (FITC) were purchased from R&D systems and 2D7 was from BD pharmingen. The HIV-1 entry inhibitors AMD3100, Maraviroc, Azidothymidine (AZT), monoclonal antibody (1C12) against CXCL12γ and monoclonal antibody that recognises the N-Terminal of SDF (K15C) were all kind gifts from Fernando Arenzana (Pasteur Institute, Paris). The antibody 1D4 was from Flint Box, University of British Columbia. Complete, EDTA-free protease inhibitor tablets were from Roche Diagnostics. Polyethylene glycol 8,000 50% w/v solution was purchased from Hampton research. Wild type human chemokines CXCL12*α*, CXCL12γ, CXCL12*α* conjugated to fluorescein isothiocyanate (FITC), M1, the C-Terminal of CXCL12γ and the biotinylated C-terminal of CXCL12γ were chemically synthesized by the Merrifield solid phase method on a fully automated peptide synthesizer using Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) chemistry as described previously (Amara, Lorthioir et al. 1999) and obtained from Françoise Baleux (Institut Pasteur, Paris, France). Antibodies against HS (10E4), chondroitin-4-sulphate and chondroitin-6-sulphate were purchased from Amsbio (Lugano, Switzerland). FITC conjugated anti-mouse antibody was purchased from Interchim (Montlucon, France).

8.1.2 Lipids and detergents

Synthetic phospholipid blend 1,2-dioleoyl-sn-glycero-3-phosphocholine/1,2-dioleoyl-sn-glycero-3-phospho-L-serine formulation (DOPC/DOPS; 7:3, w/w), the Mini-Extruder kit, filter supports and polycarbonate filters with defined pore diameter (100nm) were purchased from Avanti Polar Lipids. Detergents, n-dodecyl-β-D-maltopyranoside (DOM), 3-[(3-Cholamidopropyl)-dimethylamonio] 1-propane sulfonate/N,N-Dimethyl 3-sulfo-N-3- [3α,5β,7α,12α)-3,7,12-trihydroxy -24oxocholan-24-yl] mino]propyl] 1-propanaminium (Chaps) and Cholesteryl Hemisuccinate Tris salt (CHS) were purchased from Anatrace (see section 8.10.3.1).

8.2 Cell Culture and solubilization of co-receptors

8.2.1 Cell culture

The human chemokine receptors CXCR4 and CCR5 were over expressed in Cf2Th canine thymocyte cells as described previously (Mirzabekov, Bannert et al. 1999). Both receptors contained a C-terminal linear C9 peptide tag (TETSQVAPA) which is recognized by the 1D4 monoclonal antibody (Oprian, Molday et al. 1987). Briefly, the Cf2Th cell lines were maintained in supplemented Dulbecco's modified Eagle's medium (DMEM) from Invitrogen (Paris, France) supplemented with 10% heat-activated Fetal Calf Serum (FCS), Glutamax (2 mM) and penicillin (0.5 U/ml)/streptomycin (0.5 U/ml) antibiotics from Invitrogen (Paris, France). The Cf2Th.CCR5 expressing cell lines contained additional 500µg/ml zeocyn and 500µg/ml G418 from Invitrogen (Paris, France) and 3µg/ml puromycin from sigma (Lyon, France). Growth medium for the Cf2Th.CXCR4 expressing cell lines was additionally supplemented with 500 µg/ml G418 from Invitrogen (Paris, France). Cells were grown at 37 °C under 5% CO_2 atmosphere and detached with EDTA (Versene) purchased from Invitrogen (Paris, France).

8.2.2 Preparation of liposomes

The liposomes (final concentration of 3.3mM) were prepared as previously described (Navratilova, Dioszegi et al. 2006). Briefly, a pre-determined volume of synthetic Phospholipid Blend DOPC:DOPS (7:3, w/w) (see section 8.10.4) was transferred into a glass test tube and a thin lipid film was formed on the side walls of the glass tube by rotating the tube while evaporating all the chloroform using a stream of nitrogen gas. Once all the chloroform had been evaporated, a HEPES buffer (50mM Hepes, 150mM NaCl, pH 7.0) was added to dissolve the dry lipid films. The lipid mixture was vortexed to facilitate dissolving the films. Then the mixture was frozen, thawed, and vortexed four times. Unilamellar vesicles (ULC)/liposomes were prepared by classical extrusion through a 100nm pore diameter polycarbonate filter using an Avanti Mini-Extruder kit. Liposomes were freshly extruded for every experiment.

8.2.3 Coreceptor Solubilization

The CCR5 and CXCR4 solubilisation protocol was adapted from a described procedure (Navratilova, Sodroski et al. 2005). Briefly Cf2Th.CXCR4 expressing cells (5-8x10^6) were solubilised in 1 ml buffer consisting of 100 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 7.5), 10% glycerol, 15% PEG 8000, protease inhibitors, CHS (0.2%), DOM (1.5%), CHAPS (1.5%) and 0.33mM DOPC:DOPS liposomes The cell suspension was sonicated (6 x 1s pulses) and placed on a rotating wheel at 4°C for 3 hours. The solutions containing the solubilised coreceptors were centrifuged at 14 000 rpm for 30 minutes at 4°C and the supernatants were either used directly in SPR analysis or stored at -80°C until further use.

8.3 Protein electrophoresis and Immunodetection of Proteins

8.3.1 Immunoprecipitation

Protein G beads (Thermo Scientific) were used to immunoprecipitate either gp120 (MN or YU2) or previously solubilised GPCR (CXCR4 or CCR5). In general, protein G beads were incubated for 1 hour at room temperature under agitation with either 2-5µg of the polyclonal goat anti gp120 (D7324, Aalto Bio Reagents) or with monoclonal mouse anti C9 (1D4). The D7324 bound- and ID4 bound-beads were then washed in PBS for 30 minutes. A pre-incubated complex of 2µg gp120 with either 1µM mCD4, or 1µM HS_{12} or 1µM mCD4-HS_{12} was added to the D7324 bound beads and incubated for one hour at room temperature under gentle agitation in an incubation buffer (50mM HEPES, 5mM $MgCl_2$, 1mM $CaCl_2$, 150mM NaCl pH 7.0). For the 1D4 bounds beads, 100µl of solubilised coreceptors (either CCR5 or CXCR4) in the solubilization solution (see section 8.2.3) were added and incubated with the beads for one hour at room temperature under gentle agitation. The beads were then centrifuged to remove any unbound material and 100µl of solubilised GPCRs (either CCR5 or CXCR4) was added to the gp120-BB/HS_{12}/mCD4-HS_{12}-bound beads and a pre-incubated complex of 2µg gp120 with either 1µM mCD4, or 1µM HS_{12} or 1µM mCD4-HS_{12} was added to the 1D4-bound protein G beads. These complexes were incubated for one hour under gentle agitation and then centrifuged and washed three times in solubilization solution. After the final washing step, the beads were pelleted and resuspended in SDS- Polyacrylamide Gel Electrophoresis (PAGE) sample loading buffer (see section 8.10.1.1) and <50µl of solubilization solution and then boiled for 10 minutes at 100°C in preparation for gel electrophoresis. The beads were then pelleted and the supernatants were deposited onto the gel for electrophoresis.

8.3.2 Protein Electrophoresis

Immunoprecipitated proteins (or lipid/detergent solubilised coreceptors) were added to a quarter of the volume of 5 x sample buffer (Section 8.10.1.1), and boiled for ten minutes. Samples were resolved on 12% SDS-polyacrylamide gels according to a standard protocol (Section 8.10.1.2). Gels were then used for Western Blotting.

8.3.3 Immunoblotting (Western Blot)

Western blotting of gels was performed according to a standard protocol (described in section 8.10.2). Two primary antibodies were used during western blotting and immunoprecipitation: ID4 and D-7324 were added to the membrane for one hour at room temperature.

8.4 Removal of cell surface oligosaccharides

8.4.1 Na Chlorate treatment

Chlorate is known to be an *in vitro* inhibitor of ATP– sulphurylase, the first enzyme in the biosynthesis of PAPS (3'-Phosphoadenosine-5'-phosphosulfate), the high-energy sulphate donor in biological reactions. Chlorate competes with the sulphate ions (PAPS) that bind ATP-sulphurylase and thus affects HS biosynthesis by reducing N- and O-sulphation (Leong, Morrissey et al. 1995; Safaiyan, Kolset et al. 1999). Sodium chlorate is toxic at high concentrations for the cells, however at lower doses HS GAG chains are produced but not sulphated. Na Chlorate was freshly prepared for each cell culture treatment and cells (CEM and Cf2Th) were passaged up to three times in the presence of 30mM Na Chlorate each day. Cells were harvested and then used in fluorescence-activated cell sorter (FACS) analysis.

8.4.2 Enzymatic digestion

The following three enzymes were used in the laboratory; Heparinase I, Heparinase III and Chondroitinase ABC. Heparinase I digests the HP and HS at the link between hexosamine and O-sulfated uronic acid. Heparinase III cuts the link between the hexosamine and glucuronic acid in HS. Chondroitinase ABC digests the chondroitin sulfates A, B or C at the link between the hexosamine and uronic acid (iduronic or glucuronic). For each enzymatic digestion, $5x10^6$ cells per ml were detached from the culture flask with versene (Invitrogen) and resuspended in 500µl digestion buffer (5% fetal calf serum, 2mM $CaCl_2$ in RPMI medium) containing 50 mu Heparinase I and II and 1 U Chondroitinase ABC. The cells were incubated at 37°C for one hour under agitation, washed in PBS and then were used in FACS analysis.

8.5 Separation of dodecasaccharides

Heparan sulphate (HS) dodecasaccharides were prepared by Rabia Sadir as previously described (Sadir, Baleux et al. 2001). In order to fractionate the HS_{12} samples into sub-populations of differently sulphated oligosaccharides, the dodecasaccharides were resolved and eluted from a ProPac PA1 9 x 250mm HPLC column in NaCl at pH 3.0. Before oligosaccharide elution, the column was equilibrated in Mili Q water with the pH adjusted to 3.0 with HPLC grade HCl. A three stage linear salt gradient was performed for the elution; from 0 to 400mM NaCl for 10 mins, then from 400mM to 1.4M NaCl over one hour and then from 1.4M to 2M in 5 minutes at a flow rate of 5ml/min. To elute all oligosaccharide species from the column, 2M NaCl pH 3.0 was passed through the column for 36 minutes after each gradient. Fractions (5ml) were collected and pooled according to their absorbance profile at 232nm. The pooled fractions were desalted through multiple dialysis passages (6 changes of water of 5 hours each) in Mili Q water using dialysis membranes (Spectra POR 7 dialysis Membrane, MWCO 1000, spectra labs). After a total of 19 runs (equivalent of 95mg of fractionated HS_{12}), the desalted oligosaccharides were freeze-dried and then the amount of HS_{12} was

determined by weighing and measurement of UV absorbance at 232 nm. Oligosaccharides were stored at -20°C until further use.

8.5.1 Quantification of HS

When quantifying the amount of oligosaccharides obtained in the library, two measurements were made; that of the weight and the UV absorbance at 232nm. The latter required a standard curve and this was made using the HS_{12} that was size-separated using gel-filtration (MW 3300 g/mol). Solutions at 0, 1.25, 2.5, 5, 10, 20, 30 and 50 µM were prepared and the absorbance at 232 nm was measured in a quartz cuvette.

8.5.2 Estimation of purity

The purity of each HS_{12} fraction was assessed by running the samples in a PAGE analysis. Oligosaccharides (in H_20, 20% glycerol) were run through a stacking gel (5% acrylamide [49:1 ratio], 2% temed) at a constant voltage of 150V for one hour, then through a separation gel (30% acrylamide [19:1 ratio], 5% temed) at a constant current of 25mA for 3-4 hours. The running buffer is 25 mM Tris, 192 mM Glycine pH 8.3 and the molecular weight maker is a mixture of bromophenol blue, phenol red and Xyanol blue in 20% glycerol. Once the oligosaccharides had reached the end of the separation gel, bands were visualised by staining with 0.08% aqueous Azure A for 10 minutes and the gel was subsequently washed to remove excess stain.

8.6 Study of protein-protein interactions using surface plasmon resonance

8.6.1 CXCL12 binding to CXCR4

To study the interaction between CXCR4 and its ligands (antibodies, chemokines), CM4 sensorchips were activated with 50 µL of 0.2 M N-ethyl-N'-(diethylaminopropyl)-carbodiimide (EDC) and 0.05 M N-hydroxy-succimide (NHS) at 5 µL/min. Initially, mAb 1D4 (5 µg/mL in 10 mM acetate buffer, pH 5) was injected at 5 µL/min over one of the EDC/NHS activated flow cell until levels of approximately 7000 response units (ru) were obtained. Solubilized CXCR4 coreceptors were captured via the interaction between its C9 C-terminal tag and the ID4 immobilized on the CM4 chip surface at a flow rate of 5µl/min to yield approximately 3000 ru. Surfaces were then blocked with pH 8.5 1 M ethanolamine for 5 minutes. The solubilised CXCR4 capture was performed in the running buffer (50mM HEPES ph 7.0, 150mM NaCl, 5% glycerol, 5 % PEG 8000, 5 µM $CaCl_2$, 1 µM $MgCl_2$, 0,1 % DDM, 0,1 % CHAPS, 0,02 % CHS, 50 nM 7 :3 DOPC :DOPS, 3% DMSO and 0,2 mg/ml BSA) and then the system was left to run at 5µl/min for approximately 20 minutes to allow for reconstitution of the lipid bilayer around the captured GPCRs. Interactions between the solubilised coreceptors and their natural ligands (CXCL12α and CXCL12γ) and antibodies (12G5, 4G10 and antisulphotyrosine), were observed in real-time.

Samples were injected over both the coreceptor and control (mAb ID4) surfaces at a flow rate of 50-100 µl/min for 1 minute and 5µl/min for 12 minutes for the ligand and antibodies respectively. After each CXCL12γ and mAb injection, the 1D4 surfaces were regenerated with 10mM NaOH containing 1% *n*-octyl-β-D-glucopyranoside at 100µl/min (Navratilova, Sodroski et al. 2005). However, no regeneration was required between the different concentration injections of CXCL12α and the ligand dissociates fully from the bound coreceptors. The affinity of the CXCR4-CXCL12α interaction was determined by injecting a range of concentrations from lowest to highest (5, 10, 20, 30 and 50nM) over the CXCR4 and control surfaces so as to minimise the accumulation of the chemokine on the surface. The experiments were all performed in triplicate and binding curves were analyzed with BIAevaluation (GE Healthcare).

When performing biacore kinetic analysis, mass transport limitations and re-binding are important factors to take into account. Lower surface densities of receptor are favoured so as to decrease the rate of kinetic ligand binding and a higher flow rate is preferred so as to increase the rate of transfer of the analyte to the surface. With lower flow rates, the rate at which the surface binds the analyte may exceed the rate at which the analyte can be delivered to the surface; consequently, the measured association rate constant (k_{on}) is slower than the true k_{on}. Then when the analyte is dissociating, it can rebind to the unoccupied ligand before diffusing out of the matrix and being washed from the flow cell; consequently, the measured dissociation rate constant (apparent k_{off}) is slower than the true k_{off}. Although the dextran matrix may exaggerate these kinetic artefacts (mass transport limitations and re-binding), they can affect all surface-binding techniques.

8.6.2 Screening HS mimetic peptides

The interactions between gp120 and its ligands (CD4, mAb 17b, CCR5 and CXCR4) were analyzed by SPR technology. For that purpose, CM4 sensorchips were activated with 50 µL of 0.2 M EDC and 0.05 M NHS at 5 µL/min. Then, soluble CD4 (10 µg/mL in 10 mM acetate buffer, pH 5), streptavidin (200 µg/mL in 10 mM acetate buffer, pH 4.2), mAb 17b (5 µg/mL in 10 mM acetate buffer, pH 5) or mAb 1D4 (5 µg/mL in 10 mM acetate buffer, pH 5) were injected at 5 µL/min over one of the EDC/NHS activated flow cell until levels of 1200 (for sCD4), 700 (for mAb 17b), 3000 (for streptavidin) or 6000 (for mAb 1D4) ru were achieved. Surfaces were then blocked with pH 8.5 1 M ethanolamine for 5 minutes. In some cases, gp120 was also immobilized onto CM4 sensorchip. For this, MN (50µg/ml in 5 mM maleate buffer, pH 6) or YU2 (50µg/mL in 10 mM acetate buffer, pH 4.8) were injected at 5 µL/min over an EDC/NHS activated flow cell until levels of 4500 RU was obtained. Surfaces were then blocked with pH 8.5 1M ethanolamine for 5 minutes. Molecules under investigation were injected over the different surfaces and the binding responses were recorded as a function of time.

The 1D4 surface was then equilibrated into a running buffer consisting of 50mM HEPES pH 7.0, 150 mM NaCl, 5% glycerol, 5 % PEG 8000, 5 µM $CaCl_2$, 1 µM $MgCl_2$, 0.1 % DDM, 0.1 % CHAPS, 0.02 % CHS, 5 µM of 7:3 DOPC:DOPS and 0.1 mg/ml BSA. Solubilised CXCR4 or CCR5, in the above described liposome

preparation, were captured via the interaction between its C9 tag and the 1D4 mAb to a level of approximately 3000-5000 ru. The system was then equilibrated with the running buffer at 5 µl/min for approximately 20 minutes. Complexes of gp120 and mCD4 or inhibitors were injected at 30µl/min over the coreceptor surfaces for 2.5 minutes and the dissociation was followed for 4 minutes.

8.7 Flow cytometric analysis

For direct and competitive CXCR4 binding assays, CXCL12α, CXCL12γ, C-Terminal CXCL12γ and M1 binding was detected in both $CXCR4^+$ Cf2Th canine thymocytes and $CXCR4^+$ CEM T lymphocytes. Indirect binding experiments were performed at 4°C on a rotating wheel with fixed amounts of chemokine in the presence or absence of 1µg/ml of HP12. Briefly, cells were washed twice and suspended in buffer (1x PBS, 1% BSA, 0.02% Azide, 1mM EDTA) with 50nM of each chemokine in the presence or absence of the competitor. The cells were incubated for 1 hour and then washed twice in buffer to remove any un-bound ligands. To measure the level of cell-bound chemokine, the cells were re-suspended and incubated for 1 hour with either mAb IC12 (recognises the C-terminal of CXCL12γ) or CXCL12α which is directly conjugated to FITC. The cells were then washed two times in buffer and anti-mouse-FITC secondary antibody (1:300) was added to the cells for one hour under agitation. Finally, cells were washed two times in buffer, fixed in 4% paraformaldehyde buffer, washed a further two times and re-suspended in buffer for fluorescence-activated cell sorter FACS analysis (FACSscan, Becton Dickinson, CA). All assays were performed in triplicate and included controls with no chemokine to verify the background staining. For the verification of enzymatic digestion of cell-surface HS, the following primary antibodies were used; 10E4, anti chondroitin-6-sulphate, chondroitin-4-sulphate and anti dermatan sulphate.

8.8 Enzyme-Linked Immunosorbent Assay (ELISA)

The synthetic peptides (sulphated and non-sulphated; synthesized by Françoise Baleux), consisting of the first 29 amino acids of the CXCR4 N-terminus, were covalently coupled to the bottoms of 96-well Nunc immobilizer amino plates (Thermo Scientific). The patented photo-coupling introduces an ethylene glycol spacer and a stable electrophilic group that reacts with nucleophiles such as free amines, thiols or hydroxy groups. The peptides were diluted into a coupling buffer (100 mM Na Carbonate pH 9.6) and between 0.5 – 5 µg of peptide was immobilized in each well, and incubated at 4°C under gentle agitation overnight. The plates were washed three times with PBS. Remaining electrophilic groups are quenched by adding 10mM ethanolamine in coupling buffer for 1hour at room temperature (RT) [to introduce a hydroxyl functional group making the surface hydrophilic]. Next, the wells were saturated with PBS-3% BSA for 1.5 hrs at RT and washed 4 times in washing buffer (PBS + 0.02% Tween). Either CXCL12γ (0 - 100 nM) or the biotinylated C-terminal of CXCL12γ (0 – 500 nM) was bound to the peptides for 1.5 hours at RT in the presence or absence of 1µg/ml HP_{12}. Then each well was aspirated and washed 4 times with washing buffer. Then 5 µl/ml antibody (K15C, 4G10 or Antisulphotyrosine) was added for 1 hour at RT in

washing buffer, aspirated and wells were again washed 4 times in washing buffer. Finally 2 µl/ml of the secondary Anti-mouse Horse Radish Peroxidase (HRP) or Anti-extravidin HRP was added for 1 hour at RT. The secondary antibody was aspirated and the wells were washed 4 times with washing buffer. The HRP linked to the secondary antibodies catalysed the conversion of the substrate 3,3',5,5'-Tetramethylbenzidine (TMB) producing a light conversion from yellow to blue, which is detectable on a spectrometer (Victor, Perkin Elmer) at 450nm. The reaction is stopped by the addition of 4M H_2SO_4.

8.9 NMR

NMR spectra were obtained with a Varian 600 MHz spectrometer equipped with a cryo probe. Two-dimensional spectra (correlating protein ^{15}N and ^{1}H nuclei) of ^{15}N labelled CXCL12γ at 100 µM concentration in NMR buffer (20mM Na-Phosphate pH 5.7, 0.01% azide, 2% complete, 10% $^{2}H_2O$) were recorded. The freeze-dried N-terminal peptide of CXCR4 (sulphated and non- sulphated) was resuspended into the NMR buffer to 1 mM. CXCL12γ spectra were first read for the chemokine alone and also following addition of increasing amounts of sulphated or non-sulphated CXCR4 peptides. The following readings were taken for each peptide/CXCL12γ molar ratio: 0.1, 0.2, 0.3, 0.5, 0.75, 1, 1.5 and 2. Negligible precipitation upon addition of the CXCR4 peptide was observed. Cédric Laguri produced the ^{15}N labelled CXCL12γ, performed the NMR experiments and analysed the data.

8.10 Standard Protocols and Recipies

8.10.1 SDS-PAGE

8.10.1.1 Solutions

Protein samples were added to a sample buffer (5 x: Tris-HCl 30mM, 1% (m/v) SDS, 0.1% (m/v) bromophenol blue, 5% glycerol, pH 6.8) and heated to 100°C for 10 minutes before loading onto the gel. The sample stacking gel was prepared by adding 4.1 ml dH_2O, 750µl 1M Tris pH 6.8, 1ml 30% Acrylamide 37.5:1 and 60µl 10% SDS. Just prior to pouring the stacking gel, 60µl 10% APS and 60µl TEMED were added to the solution. The running gel was prepared as follows; 4.9 ml dH_2O, 3.8ml 1.5M Tris pH 8.8, 6ml 30% Acrylamide 37.5:1 and 150µl 10% SDS were combined. Just prior to pouring the gel, 150µl 10% APS and 10µl TEMED were added to the solution. A pre-stained protein ladder maker (Euromedex) was used to follow the migration of the samples and to estimate the molecular weight of the proteins.

8.10.1.2 Resolving SDS-PAGE gels

The stacking gel was migrated at 20mA and then the separation/running gel migrated under a constant current of 25mA in a running buffer (25mM Tris-HCl, 192mM glycine, 0.1% SDS) until the dye front was approximately 0.5 cm from the bottom of the gel. The gel was subsequently used in a western blot transfer and probed with immuno-marking antibodies.

8.10.2 Western Blot

8.10.2.1 Solutions

After running the SDS-PAGE, the gel was directly transferred to a PVDF membrane (Sequi-blot PVDF Membrane, 0.45µm – Millipore) for 2 hours at a constant voltage (50V) at 4°C in a transfer buffer (25mM Tris-HCl, 192mM Glycine). Following protein transfer, samples were blocked for one hour shaking at room temperature or overnight at 4°C in 5% (m/v) fat free milk powder in TBS (50mM Tris-HCl, 150mM NaCl, pH 7.4). Three 10-minute washes in TBS-tween (TBS + 0.5% Tween) followed before addition of the primary antibody.

8.10.2.2 Transfer

After incubation with the primary antibody (as described in section 8.3.3), the membrane was washed three times for ten minutes in T-TBS. Depending on the primary antibody, the secondary antibody used was either anti-mouse in the case of the mouse monoclonal antibodies, or anti-goat in the case of the anti-gp120 (D7324) and was added at a concentration of 1:7000 or 1:3000 respectively diluted in TBS and laid flat on the membrane for one hour. After this incubation, the membrane was again washed three times for 10 minutes in TBS-tween before protein detection. Bound secondary antibody was detected on Western Blot membranes using enhanced chemiluminescence (ECL). A commercial substrate (ECL Kit Amersham biosciences, GE Healthcare), was used according to the manufacturer's instructions and the peroxydase catalyses the liberation of light. After the enzymatic reaction, membranes were exposed to radiographic films (Hyperfilm^{TM} ECL, Amersham, GE Healthcare) for varying amounts of time, depending on the signal strength, before developing and fixing.

8.10.3 Detergents and Lipids

8.10.3.1 Detergents

Detergents are amphiphiles which means that they are molecules that have two different polarities, a hydrophobic polar side and a hydrophilic polar side. Surfactants and detergents (molecules that are capable of solubilising fats) are often used when studying membrane proteins. Due to their different structures, detergents can be classified into one of four different categories; non-ionic, ionic, zwitterionic and steroids.

The work presented here made use of three detergents, n-Dodecyl-β-D-maltoside / n-Dodecyl-β-D-maltopyranoside (DOM), 3-[(3-Cholamidopropyl) -dimethyl-ammonio]-1-propane sulfonate/ N,N-Dimethyl -3-sulfo-N- [3-[[3α,5β,7α,12α)-3,7,12-trihydroxy- 24-oxocholan-24-yl] amino]propyl] -1-propanaminium hydroxide (CHAPS) and Cholesteryl Hemisuccinate Tris Salt (CHS).

DOM is a non-ionic detergent with a hydrophilic head and due to its short hydrocarbon chain (C_7-C_{10}), it is a soft and non-denaturing detergent and often used when working with membrane proteins.

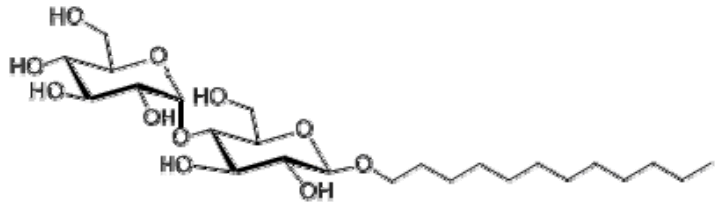


Figure 8.1 Chemical Structure of DOM: MW = 510.6 g/mol, cmc = 0.17 mM (0.0087%), Aggregation number = 78-149

CHAPS is zwitterionic detergent possessing both ionic and non-ionic properties and it is very efficient for membrane solubilization, however, it is known to denature proteins.

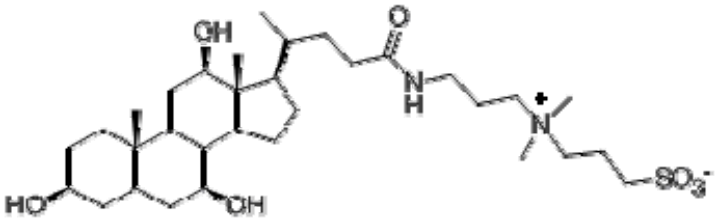


Figure 8.2 Chemical structure of CHAPS: MW = 614.9 g/mol, cmc = 8 mM (0.49%), Aggregation number = 10

CHS is a cholesterol steroid and has weaker denaturing properties with a hydrophilic head.

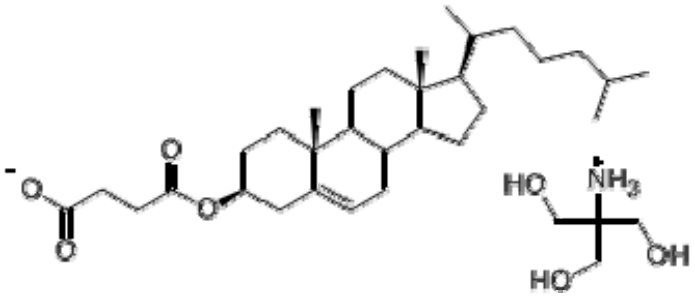


Figure 8.3 Chemical structure of CHS: MW 607.9 g/mol

8.10.4 Lipids

A synthetic phospholipid blend of 1,2-dioleoyl-sn-glycero-3-phosphocholine [DOPC] and 1,2-dioleoyl-sn-glycero-3-phospho-l-serine [DOPS] [7:3, w/w] was purchased from Avanti Polar Lipids. Incorporating Lipids into the solubilization cocktail helped to stabilize the GPCR tertiary structure.

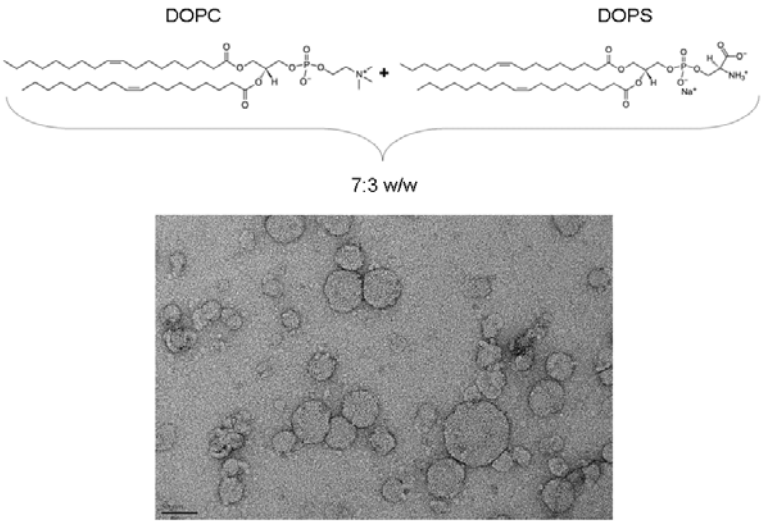


Figure 8.4 Chemical structure of DOPC and DOPS together with an electron micrograph taken of the extruded 100nm liposomes (DOPC:DOPS 7:3 [w/w]), 0.26mg/ml in 50mM HEPES 150mM NaCl

8.10.5 SPR

8.10.5.1 The SPR Principle

The biomolecular interaction analysis (BIAcore) uses the optical phenomenon of SPR in thin metal films under total internal reflection to monitor interactions between biomolecules. The resonance angle or output signal (expressed in Response Units) of the incident monochromatic p-polarized light (near infra-red) can be mathematically derived to represent the changes in the mass concentration of macromolecules in contact with the biospecific interface. Essentially, a sensor chip is a glass slide, coated with a thin gold film (due to its chemical inertness), to which a carboxymethyl dextran matrix is covalently attached. The dextran layer has a net negative charge which assists proteins (positively charged in buffers with a pH below their pI) to be electrostatically attracted to the dextran.

When a beam of incoming light passes from a material with a high refractive index (e.g. glass) into material with a low refractive index (e.g. water) some light is reflected from the interface. When the angle at which the light strikes the interface (the angle of incidence or θ) is greater than the critical angle (θ_C), the light is completely reflected (total internal reflection). If the surface of the glass is coated with a thin film of a noble metal (e.g. gold), this reflection is not total; some of the light is 'lost' into the metallic film. There then exists a second angle greater than the critical angle at which this loss is greatest and at which the

intensity of reflected light reaches a minimum or 'dip'. This angle is called the surface plasmon resonance angle (θ_{SPR}). This is due to the oscillation of mobile electrons (or 'plasma') at the surface of the metal film. These oscillating plasma waves are called surface plasmons. When the wave vector of the incident light matches the wavelength of the surface plasmons, the electrons 'resonate', hence the term surface plasmon resonance.

The 'coupling' of the incident light to the surface plasmons results in a loss of energy and therefore a reduction in the intensity of the reflected light. An evanescent (decaying) electrical field associated with the plasma wave travels for a short distance (~300 nm) into the medium from the metallic film. Because of this, the resonant frequency of the surface plasma wave (and thus θ_{SPR}) depends on the refractive index of this medium. If the surface is immersed in an aqueous buffer (refractive index or μ ~1.0) and protein (μ ~1.33) binds to the surface, this results in an increase in refractive index which is detected by a shift in the θ_{SPR}. The instrument uses a photo-detector array to measure very small changes in θ_{SPR}. The readout from this array can be viewed on the BIAcore as 'dips'. The change is quantified in resonance units or response units (RUs) with 1 RU equivalent to a shift of 10^{-4} degrees which correlates to 1pg of protein/mm^2. Apart from the refractive index, the other physical parameter which affects θ_{SPR} is temperature. Thus a crucial feature of any SPR instrument is precise temperature control.

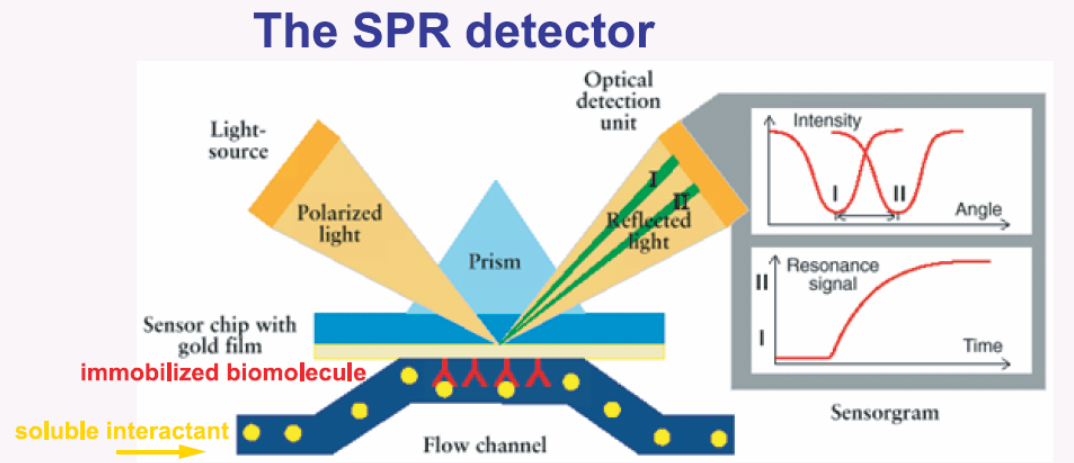


Figure 8.5 Binding is measured as a change in the refractive index as the sensor surface. A change of 0.001 degrees is equivalent to 1pg of protein bound per mm^2.

8.10.5.2 Amine Coupling

A standard protocol for amine coupling was used. The first step is to activate the carboxymethyl groups of the dextran with a mixture of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS), thus creating a highly reactive succinimide ester which reacts with the primary amine and other nucleophilic groups on proteins to form a covalent bond. The second (coupling) step is to inject the protein in a buffer with a pH lower that the protein's pI, thereby driving the coupling reaction. The third (blocking) step, blocks the remaining activated carboxymethyl groups by injecting very high

concentrations of 1M ethanolamine-HCl pH 8.5. The high concentration of ethanolamine also helps to elute any non-covalent bound material. The surface is then ready to be used for further binding experiments and can be regenerated by a specific regeneration solution which will remove any bound analyte, and the ligand that is covalently coupled will remain coupled to the dextran surface.

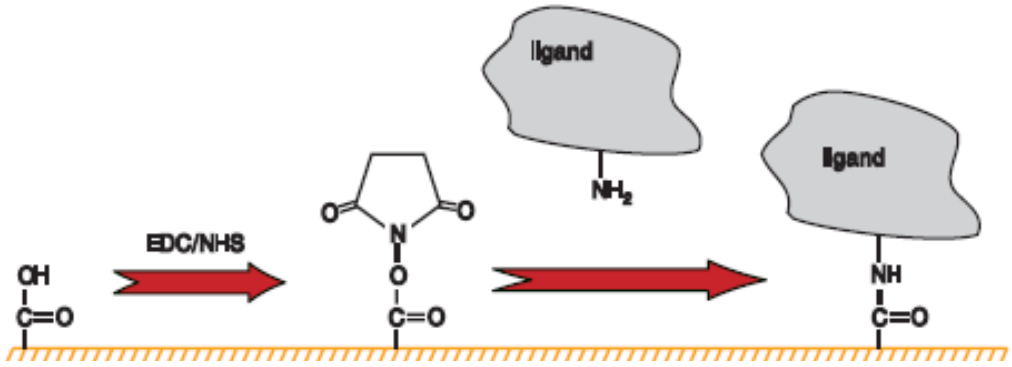


Figure 8.6 Amine coupling ligands to the surface using EDC/NHS.

C. DISCUSSION

Chapter 9: Conclusions and Perspectives

9.1 Conclusions et Perspectives (sommaire en français)

Il y a plus de 10 ans, la purification des GPCR de leurs milieux naturels a souvent abouti à des protéines dépourvues de leur conformation native, ce qui rendait leur utilisation dans des dosages fonctionnels totalement futile (Gether and Kobilka 1998; Ji, Grossmann et al. 1998). Ainsi, l'étude des GPCRs a été limitée aux tests cellulaires. Toutefois, en raison de l'hétérogénéité de la surface des cellules, l'interprétation des résultats de liaison est souvent compliquée. Ceci est particulièrement problématique pour les études impliquant des récepteurs de chimiokines parce que leurs ligands (qu'ils soient naturels ou viraux) se lient à d'autres molécules de la surface cellulaire, y compris des HS. Ainsi, la surexpression, solubilisation et purification des protéines pour l'analyse de liaison, est une option beaucoup plus attrayante pour mieux comprendre la structure et la fonction des GPCRs. La Resonance Plasmonique de Surface (SPR) est une technologie innovante pour l'étude des interactions biomoléculaires en temps réel sans aucun étiquetage. Nous avons cherché à améliorer la spécificité et l'affinité d'un inhibiteur bivalent de l'entrée du VIH-1 qui existe déjà (Baleux, Loureiro-Morais et al. 2009). Pour ce faire, nous avions besoin d'une plateforme qui nous permettrait d'immobiliser les corécepteurs de VIH-1 natifs (CCR5 et CXCR4) et de cribler différentes molécules pour leur capacité à inhiber la liaison des complexes gp120-CD4 aux corécepteurs immobilisés. Ici, on a donc immobilisé des récepteurs de chimiokines solubilisés et comme contrôle de ce processus, nous avons étudié des chimiokines liant ces corécepteurs. Ce système étant fonctionnel, nous avons pu également l'utiliser pour mieux comprendre la régulation de la fonction des chimiokines par les GAGs. Une fois que nous étions sûrs que les corécepteurs étaient fonctionnels, nous avons criblé des molécules qui inhibent la liaison du complexe gp120-CD4 aux corécepteurs afin de concevoir des molécules plus efficaces qui peuvent empêcher l'utilisation du corécepteur et l'entrée des souches primaires du VIH-1.

9.1.1 Etude de la liaison de CXCL12 à CXCR4

L'efficacité de la liaison des anticorps (12G5, 4G10 et anti-sulphotyrosine), des chimiokines (CXCL12α et γ) et de l'enveloppe virale (X4 gp120) à CXCR4 solubilisé et immobilisé suggère fortement que la protéine CXCR4 conserve sa conformation native. La première partie de ce travail (chapitre 5) est une étude qui a porté sur l'utilisation de CXCL12 comme ligand pour le CXCR4 solubilisé et immobilisé afin de confirmer son intégrité fonctionnelle. CXCL12 est le ligand naturel pour CXCR4 et interagit avec CXCR4 par la liaison de l'extrémité N-terminale et des deuxième et troisième boucles extracellulaires. Nous avons été en mesure de déterminer l'affinité de CXCR4 pour CXCL12α qui a donné un Kd de 13 ± 1,6 nM, ce qui est cohérent avec des valeurs publiés antérieurement. Pour CXCL12γ, l'analyse préliminaire des courbes montre que l'association et les constantes de vitesse de dissociation ont été dominées par le transfert de masse. Ici, l'isoforme CXCL12γ montre une plus grande affinité pour le CXCR4 immobilisé et cela signifie que CXCL12γ est un ligand plus fort pour le CXCR4

que CXCL12α. Comme la seule différence entre les isoformes CXCL12α et CXCL12γ est l'extension C-terminale, ces résultats suggèrent que c'est l'extension C-terminale, non-structurée qui est responsable de l'affinité augmentée de CXCL12γ pour CXCR4. Le mutant M1 (9 acides aminés basiques ont été mutés dans son extrémité C-terminale) a été un intermédiaire entre les deux et, de façon intéressante, M1 montré un profil de liaison pour le CXCR4 proche de celui de CXCL12α. Ainsi, le fait que CXCL12γ a une plus grande affinité pour le CXCR4 et qu'il ne se dissocie pas du corécepteur est lié au nombre de résidus basiques présent dans le domaine C-terminal. Puisque selon nos données, la haute affinité montré par CXCL12γ pour CXCR4 est due à l'extrémité C-terminale basique, on devrait être en mesure de rivaliser la liaison à CXCR4 par la présence de GAGs anioniques, qui ont été montrés capables de lier le C-terminal de CXCL12γ.

Un oligomère 12mer d'HP (1μg/ml) n'a eu aucun effet sur la liaison de CXCL12α ou M1 à CXCR4, que l'analyse soit faite par SPR ou par cytométrie de flux des cellules. Cependant, des oligosaccharides de même taille et de même concentration ont en effet eu un effet drastique sur la liaison de CXCL12γ sur le CXCR4. Nous avons supposé que la queue C-terminale basique et allongée de CXCL12γ peut se lier au N-terminal anionique de CXCR4 qui possède jusqu'à trois résidus tyrosine, qui peuvent chacune être sulfatées, contribuant à la forte affinité observée entre CXCL12γ et CXCR4. Compte tenu de la complémentarité entre la charge de surface des sulfates dans le N-terminal de CXCR4 et le C-terminal très basique de CXCL12γ, il était logique de proposer que la grande affinité qui est observée entre CXCL12γ et CXCR4 est due à l'interaction ionique entre les acides aminés basiques dans le C-terminal de CXCL12γ avec les résidus tyrosines sulfatées présents dans le domaine N-terminal de CXCR4. Nous avons également été en mesure de bloquer la liaison d'un anticorps anti-sulfotyrosine sur l'extrémité N-terminale de CXCR4, en présence de CXCL12γ comme autre preuve de cette interaction. Toutefois, afin de tester l'hypothèse que le C-terminus de CXCL12γ se lie à des sulfotyrosines dans la partie N-terminale de CXCR4, des approches structurales et cellulaires ont été employées. Pour la RMN, CXCL12γ marqué au ^{15}N a été titrée avec des ajouts progressifs de peptide N-terminal sulfaté ou non sulfaté de manière à identifier les résidus impliqués dans la liaison du peptide sulfaté. Cette expérience de RMN a produit des données suggérant fortement que la présence de sulfates, permet la liaison du peptide N-terminal de CXCR4 au C-terminal du CXCL12γ. Ainsi, la plus grande affinité observée entre CXCL12γ et CXCR4 est due à la partie C-terminale de la chimiokine.

Nous proposons l'hypothèse qu'en raison de la forte affinité entre CXCL12γ et le N-terminal de CXCR4, cette chimiokine est «captive» sur la partie N-terminale du corécepteur et serai spatialement trop loin du site d'activation ; elle est donc une chimiokine avec une plus faible signalisation. Conformément à l'hypothèse que la plus grande affinité que CXCL12γ a pour CXCR4 est due à l'interaction des acides aminés basiques dans le C-terminus de CXCL12γ avec les tyrosines sulfatées dans le N-terminus de CXCR4 – des expériences d'activation de ERK 1/2 avec des chimiokines (CXCL12α, CXCL12γ et M1) en présence et en absence d'oligosaccharides ont été effectuées. Nous avons montré que en présence de 10 μg/ml d'héparine, CXCL12γ induit une activation 4X plus forte de ERK 1/2, alors

il n'y a pas de différence d'activation de ERK 1/2 pour CXCL12α et M1, en présence d'héparine. Dans notre modèle, en présence de courts oligosaccharides sulfatés, l'interaction CXCL12γ-N-terminal avec CXCR4 est déstabilisée permettant de détacher la chimiokine et d'accéder au site d'activation dans les boucles extracellulaires de CXCR4, donc l'héparine soluble pourrait améliorer la signalisation de CXCL12γ. D'autres hypothèses pourraient être proposées telles que: l'héparine soluble est capable de stabiliser l'extrémité C-terminale désordonnée de CXCL12γ, lui permettant ainsi de signaliser plus efficacement avec moins de liaison non spécifiques. Des expériences supplémentaires doivent étudier si oui ou non CXCL12γ dimerise en présence du GAG, comme le fait CXCL12α, ou si l'extrémité C-terminale basique de CXCL12γ éviterait la dimérisation? Nous émettons l'hypothèse que CXCL12γ est exprimée et sécrétée sous forme de petits gradients haptotactiqus très concentrés et localisés pour la séquestration des cellules immunitaires sur une période beaucoup plus prolongée, par opposition aux grands gradients étalés créées avec CXCL12α qui auront une durée de vie plus courte, en comparaison. Une autre hypothèse pour la diversité fonctionnelle des CXCL12γ est celui de la dégradation protéolytique. Vu que CXCL12γ montre une fréquence inférieure d'activation de CXCR4 due à sa séquence C-terminale, un niveau de régulation de la signalisation de CXCL12γ pourrait être par la digestion de l'extrémité C-terminale?

9.1.2 L'étude d'un inhibiteur d'entrée

Afin de cibler l'entrée du VIH-1, un inhibiteur efficace devrait être capable de bloquer non seulement de liaison avec CD4 mais aussi la liaison de gp120 à son corécepteur (idéalement aux deux corécepteurs CCR5 et CXCR4). C'est une tâche particulièrement difficile parce que les surfaces de la glycoprotéine du VIH-1 qui sont impliqués dans l'entrée sont soit cryptique (le site CD4i) soit bien cachée par un bouclier glycosylé. Il ya beaucoup de médicaments antirétroviraux qui existent, dont un inhibiteur d'entrée (seulement contre les virus R5) et un inhibiteur de fusion, mais il n'y a toujours pas de médicaments qui peuvent inhiber efficacement l'infection par le VIH-1. Il ya un grand besoin d'un inhibiteur d'entrée qui se lie au virus, qui ait une faible toxicité et inhibe à la fois les variants R5 et X4 de VIH-1. Dans ce but, nous avons cherché à augmenter la spécificité et l'affinité d'une molécule bivalente (prototype) qui inhibe l'entrée, mCD4-HS_{12}. En raison de la nature extrêmement complexe et hétérogène des HS (Esko et Lindahl, 2001), sur la base des 48 combinaisons disaccharidiques différentes, une banque de 12 mer ($48^6 = 10^{10}$) serait inconcevable à faire synthétiser pour des études structure-fonction. De plus, l'assemblage du 12mer utilisé dans le prototype a pris 1 an à synthétiser et donc une plus grande banque de 12mers différemment sulfatés serait impossible à produire. Ainsi, la partie glycosidique de ce prototype devait être améliorée / optimisée et il y aurait deux façons de faire cela : une approche aurait été de fractionner une banque d'oligosaccharides naturels 12mer héparane sulfate par chromatographie échangeuse d'ions, de purifier et de tester chaque fraction pour sa capacité à inhiber laliaison de la gp120-CD4 au corécepteur, puis de purifier la fraction la plus active en grandes quantités. Cette approche serait fastidieuse et aussi ne se prêterait pas à une analyse structure-fonctionnelle parce que cette technique de

purification est loin d'être précise et qu'une solution homogène d'un type de structure 12mer ne serait pas possible d'obtenir. Même si un oligosaccharide 12mer avec une affinité de liaison élevée pour la gp120 avait été identifié, le séquençage de ce 12mer pour identifier sa structure disaccharidiques exacte serait aussi très fastidieux.

La seconde approche a été la conception de peptides qui miment les HS, contenant des acides aminés chargés négativement qui imitent les fractions HS et cela simplifierait le processus de deux manières; les peptides sont simples à synthétiser et une séquence peptidique homogène se prêterait à une analyse séquence/activité. Pour imiter les groupes fonctionnels hydroxyles, carboxyles et sulfates trouvés sur des fragments d'HS, les peptides contenant des sérines, asparagines et tyrosines (sulfatées ou non sulfatées) ont été produits. La séquence $S(XDXS)_3$ a été utilisée où X représente un acide aminé et au final cinq peptides ont été synthétisés; P3Y (où X = tyrosine non sulfatée), $P3YSO_3$ (où X = sulphotyrosine), P3Asu (où X = acide aminosuberic), P3pF (où X = p-carboxyméthyl phénylalanine) et E13 (où toute la longueur est de l'acide glutamique - un polyanion non spécifique). Les peptides contenant la sulphotyrosine ($P3YSO_3$) ont montré une capacité à bloquer l'anticorps monoclonal 17b avec une IC_{50} de 3 µM, qui se compare bien aux molécules déjà publiées.. Lorsque $P3YSO_3$ a été couplé de façon covalente à mCD4, un effet synergique a été vu et quantités de quelques nM de mCD4-$P3YSO_3$ étaient nécessaires pour empêcher gp120-CD4 de se lier au mAb 17b. Vu que mAb 17b n'est qu'un mime du corécepteur, la capacité d'inhibition du mCD4-$P3YSO_3$ devait être testée sur CCR5 et CXCR4 fonctionnels et solubilisés immobilisés sur des surfaces BIAcore. En utilisant le protocole de solubilisation des GPCR qui avait été mis en place et optimisé dans le chapitre 5, CCR5 et CXCR4 ont été immobilisés sur la surface et les interactions entre la gp120, en présence et en absence de mCD4 ont été enregistrées. Ce travail montre que conjugué à mCD4, $P3YSO_3$ interagit avec les deux gp120 (R5 et X4), alors que HS_{12} est surtout actif vis-à-vis des gp120 X4.

Nous avons obtenu une affinité de 154 ± 68 nM pour l'interaction gp120/CD4 - CXCR4 ce qui est comparable à celle obtenue dans un système cellulaire (Doranz, Baik et al 1999; Babcock, Mirzabekov et al 2001.), et les valeurs de 11,5 ± 2,9 nM pour l'interaction gp120/CD4-CCR5, également en accord avec celles trouvées par une technique similaire (Navratilova, Sodroski et al. 2005). Sur les 5 peptides bivalents qui ont été criblés sur les surfaces GPCR, mCD4-$P3YSO_3$ a été le seul peptide qui inhibait complètement la liaison entre gp120 et les deux corécepteurs CCR5 et CXCR4 avec une stoechiométrie 1:1. Curieusement, les autres peptides inhibiteurs bivalents de l'entrée du VIH-1 (mCD4-PSY, mCD4-P3Asu, mCD4-P3pF et mCD4-E13) ont tous montré à des degrés divers, des activités antivirales dans l'expérience d'infection des PBMC par la souche LAI (au tropisme X4) du VIH-1. Cependant, ils étaient tous inefficaces contre la souche Ba-L (tropisme R5). Ainsi, ces autres peptides ne sont pas redondants, ils peuvent également être envisagées comme des thérapies antivirales qui sont administrées comme traitement de sauvetage, en plus de composés contre des virus à tropisme R5 (par exemple, le maraviroc) pour les patients qui ont le virus utilisant CXCR4 ou sont infecté par une souche de VIH utilisant un double tropisme. Non seulement ce peptide sulfaté représente une possible nouvelle génération d'inhibiteur d'entrée du

VIH-1, mais il ouvre également une toute nouvelle stratégie pour la détermination de la relation structure-fonction entre les molécules sulfatées et des protéines cibles. Cette nouvelle molécule, mCD4-P3YSO$_3$, a une IC_{50} aussi basse que 1nM et mérite donc d'être testée pour son efficacité dans un modèle animal chez des macaques dans le modèle SHIV du VIH. Dans approche préventive, mCD4-P3YSO$_3$ peut être incorporé dans un gel qui sera appliqué sur la muqueuse vaginale et anale et donc être utilisé en tant que microbicide. Dans ce cas, la distribution de la drogue sera locale et concentrée sur la muqueuse du tractus génital - le premier site d'entrée lors de la transmission sexuelle du virus. Cependant, si mCD4-P3YSO$_3$ est utilisé comme un traitement, il devra être administré par voie intraveineuse ou sous-cutanée à intervalles réguliers pour inhiber la dégradation possible ou l'hydrolyse de la molécule par des protéases d'hôte. Ainsi la distribution du médicament dans le sang dépend de la régularité des injections. Le mCD4-P3YSO$_3$ est dépourvu de toxicité jusqu'à 1 µM en culture cellulaire, ce qui augure bien pour les tests de toxicité dans le modèle animal.

En termes de stabilité de mCD4-P3YSO$_3$, une approche qui pourrait être utilisée pour protéger la partie peptide anionique de la molécule bivalente contre l'hydrolyse par les protéases de l'hôte, serait d'utiliser les stéréoisomères de chaque acide aminé. Les acides aminés D sont trouvés dans la nature, cependant, les acides aminés L sont principalement utilisés pour former des protéines dans les organismes (les acides aminés D sont trouvés dans la peau des grenouilles, en particulier). Vu que la reconnaissance de la cible pour la partie anionique de la molécule bivalente n'est pas strictement dépendante de la structure (comme c'est le cas pour la partie mCD4), des isomères D pourraient être utilisés car ils sont encore fonctionnels en plaçant une charge négative à une certaine position, indépendamment de leur chiralité. Ainsi, les isomères-D ne seront pas reconnus par l'hôte et ne seront donc pas dégradés par les protéases de l'hôte. Concernant la production de mCD4-P3YSO$_3$, comme mentionné précédemment, le prototype mCD4-HS$_{12}$ a pris un an à synthétiser.. Notre inhibiteur d'entrée bivalent de seconde génération est donc déjà beaucoup plus rapide à synthétiser par rapport au prototype et mCD4-P3YSO$_3$. Dans un effort d'optimisation, le peptide sulfaté peut être remplacé par d'autres forme de molécules anioniques. On pourrait remplacer le sulfate (SO_4^{2-}) sur les résidus tyrosines par du sulfonate (SO_2O^-), qui ne diffèrent que par un seul atome d'oxygène et sont techniquement beaucoup plus faciles à ajouter à une chaîne peptidique pendant la synthèse. Toutefois, ces nouveaux composés devront être examinés pour déterminer leur capacité à inhiber la liaison des complexes gp120-CD4 aux corécepteurs de l'hôte. Notre inhibiteur d'entrée bivalent (mCD4-P3YSO$_3$) vise à la fois les variants R5 et X4 du VIH-1 et cette molécule est peu susceptible d'induire des mutations dans l'enveloppe pour échapper à la liaison, puisque le développement de mutations dans deux parties différentes de la protéine simultanément est probablement difficile pour le virus. Toutefois, si la résistance se développe à cet inhibiteur il pourrait être utilisé en combinaison avec d'autres antirétroviraux ou en tant que traitement prophylactique.

La prochaine étape de notre projet est de cristalliser le complexe formé par mCD4-P3YSO$_3$ avec la gp120 afin que les résultats puissent en être utilisés pour

déterminer le site exact de liaison entre mCD4-P3YSO$_3$ et gp120. A partir de ces résultats, un inhibiteur ayant une affinité encore plus élevée peut être conçu contenant uniquement les sulfotyrosines essentielles impliquées dans l'interaction, dont le nombre peut être inférieur à celui se trouvant dans mCD4-P3YSO$_3$. Finalement, un trimère mCD4-P3YSO$_3$ peut être modélisé et produit, ce qui sera difficile d'un point de vue synthétique, mais très probablement plus efficace pour bloquer l'entrée virale parce qu'il va se lier à l'ensemble de l'enveloppe trimère au lieu d'un monomère enveloppe. Ce composé ne présente aucune toxicité jusqu'à 1 μM et inhibe l'entrée par CCR5 et CXCR4 en utilisant des virus de laboratoire adaptés (LAI [X4] et Ba-L [R5]) ainsi que les souches virales primaires à partir de divers sous-types avec une ED$_{50}$ de 1 nM. Le mCD4-P3YSO$_3$ a montré un ED50 faible (0,2 - 1,2 nM) pour 5 isolats cliniques primaires (clade A X4 [92UG029], clade B R5 [SF162], X4R5 clade B [92US723], X4R5 clade B [96USHIPS4], X4 clade B [92HT599]) et il apparaît une ED$_{50}$ de 29 nM pour un virus clade C [98IN017] à tropisme X4. Cette activité antivirale à tropisme double est sans précédent pour tout autre inhibiteur d'entrée du VIH-1.

9.2 Setting up the GPCR Immobilization Platform

Interactions between membrane-bound receptors and their ligands have been studied by incorporating GPCRs into retrovirus particles and capturing these particles on a biosensor surface (Hoffman, Canziani et al. 2000). Despite the advantages of not having to solubilize, purify and reconstitute the membrane proteins into liposomes, this technique presents many complications; the quantity of functional GPCRs incorporated into the viral particles cannot be regulated, the surface for potential non-specific binding is increased due to the nature of the large spherical particles and a lower density of GPCRs is present on the biosensor surface due to their immobilization within the viral particles. Another disadvantage of this approach is that when the surface is regenerated with a regeneration solution, there is a risk that the GPCRs imbedded within the viral membrane become denatured and/or damaged and are thus not optimal for further binding experiments.

Other techniques such as the use of paramagnetic proteoliposomes (PMPLs) have been described, which allow the study of membrane-associated solubilized native GPCRs (Mirzabekov, Kontos et al. 2000; Babcock, Mirzabekov et al. 2001; Devesa, Chams et al. 2002). Essentially, the solubilized GPCRs are captured on a magnetic bead via an affinity tag and following this, a liposome is reconstituted during detergent removal. Proteoliposomes, on the other hand do not have a central magnetic bead; they are essentially liposomes that have been formed by detergent removal through dialysis in the presence of purified CD4 and CXCR4 (Zhukovsky, Basmaciogullari et al. 2010). Despite the advantage of the CD4 and CXCR4 molecules being able to move laterally within the membrane, which is not the case for PMPLs, they are randomly oriented in either direction when incorporated into the proteoliposomes and thus the quantity of functional molecules could be halved. This technique allows for interaction studies between host membrane proteins and viral envelopes as well as thermal stability studies on the HIV-1 coreceptors, however, optimized efficiency is required due to the high non-specific binding between proteoliposomes and cell membranes (Zhukovsky, Basmaciogullari et al. 2010).

Surface Plasmon Resonance (SPR) is an innovative technology for studying biomolecular interactions in real-time without any labeling (Fagerstam, Frostell-Karlsson et al. 1992). As opposed to structural techniques such as X-ray crystallography, NMR, electron microscopy and sequence analysis, SPR is a technique that gives detailed information on the dynamic interaction between molecules/proteins, providing more than just a snapshot of the interaction frozen in time. Other techniques such as affinity chromatography, immunological techniques and isothermal titration calorimetry, are able to obtain valuable information on the conditions and specificity of the interaction. However these techniques are unable to use such low quantities of unlabelled protein. Often, large quantities of purified protein are required as well as a form of labeling in spectrophotomeric techniques, and the labeling could interfere with the binding/active-site of the protein. However, the SPR technique may not require protein purification or labeling of any kind and is highly useful for weak and strong biological interactions. Unlike radioligand assays, SPR is able to determine

the amount of active receptor that has been immobilized on the surface. This is indicated by the amount of analyte binding to the immobilized ligand on the surface. The ligand of interest is covalently coupled (either directly, or a capturing molecule is first covalently coupled to the surface) using amine-coupling chemistry (see Section 8.10.5.2) to the hydrophilic dextran. The analyte is injected over the immobilized ligand and the interaction is observed by monitoring the change in resonance signal and kinetic information on the interaction derived from the rate of change of the signal (see section 8.10.5.1).

SPR biosensors have been used for years to measure kinetics and affinities of molecular interactions, mostly involving soluble hydrophilic proteins. However, recently, the biosensor has been used as a tool to capture membrane solubilized GPCRs (Fagerstam, Frostell-Karlsson et al. 1992; Navratilova, Sodroski et al. 2005; Navratilova, Dioszegi et al. 2006). The advantages of such a binding assay are numerous; it can be used as a tool to screen GPCR function under different solubilization conditions, to better understand the mechanism of chemokine interactions with GPCRs, assessing the ability of molecules to target the coreceptor binding site of gp120 and it can eventually be used to screen for inhibitors of HIV-1 entry.

More than a decade ago, purification of GPCRs from their natural environments often resulted in proteins devoid of their native conformation, rendering their use in functional assays completely futile (Gether and Kobilka 1998; Ji, Grossmann et al. 1998). Thus, studying GPCRs was restricted to cell-based assays. However, due to the heterogeneity of the cell surface, often the interpretation of binding results is complicated. This is particularly problematic for studies involving chemokine receptors as their ligands bind to other cell-surface molecules including HS. Thus, over-expression, protein solubilization and purification for binding analysis, is a much more attractive option to better understand GPCR structure and function.

In the context of the objectives of this study, we sought to improve upon the specificity and affinity of a pre-existing bivalent HIV-1 entry inhibitor (Baleux, Loureiro-Morais et al. 2009). In order to do this, we required a platform whereby we could immobilize the native HIV-1 coreceptors (CCR5 and CXCR4) and screen different molecules for their ability to inhibit gp120-CD4 complexes from binding to the immobilized coreceptors.

Thus for the purpose of our studies, we decided to use SPR whereby C-terminal tagged HIV-1 coreceptors were solubilized and immobilized in an orientated fashion so that they could interact with their ligands. Here, we reduced the amount of variables that could cause non-specific binding (i.e. incorporation of cell membranes and other cell expressed membrane proteins) and we took advantage of the relatively small amount of material that is required for Biacore experiments.

The Cf2Th-CCR5 and Cf2Th-CXCR4 cell lines which were obtained from the NIH AIDS research and reference reagent program, stably express their respective coreceptors and each coreceptor possesses a C-Terminal 9 amino acid (C9) tag. Owing to the high affinity interaction between the C9 Tag and the 1D4 antibody which is covalently attached to the biosensor surface, we were able to capture the solubilized coreceptors within the apparatus. Thus, we chose to solubilize the

HIV-1 coreceptors and immobilize them on the SPR surface as this technique did not require any laborious purification steps nor any handling of radioactive material and there is a well-equipped Biacore platform at the IBS institute.

We have shown that with a solubilization cocktail (containing three detergents and a phospholipid blend, adapted from Navratilova *et al.,* 2006), both CCR5 and CXCR4 are solubilized, maintaining their functionality. Refining the solubilization protocol for these two coreceptors served as the foundation to the two studies which were conducted using this immobilized coreceptor platform:

1. Initially, in order to verify that the coreceptors retained their functionality after the solubilization process and immobilization, we required a positive control. Thus we decided to study the binding of the natural chemokine ligand, CXCL12, to CXCR4 in order serve as the proof of concept for this technique. This work constitutes the study of CXCL12α and CXCL12γ binding to CXCR4 and the role played by soluble HS.
2. The second part of this work constitutes an extensive study whereby novel entry inhibitor molecules were screened for their ability/efficiency to inhibit the gp120/CD4 – coreceptor interaction (for both CCR5 and CXCR4).

Here we immobilized solubilized chemokine receptors to study chemokine binding both in the absence and presence of soluble GAGs and to screen for molecules that inhibit gp120-CD4 binding to the coreceptors. We aimed to better understand the regulation of chemokine function by GAGs and to design more effective molecules that may prevent coreceptor utilization and entry of primary HIV-1 strains.

9.2.1 Study of CXCL12 Binding to CXCR4

The first objective of this work was to set up a functional surface of solubilized GPCRs whereby binding interactions could be studied in real time and kinetic data could be derived from the interaction curves. We initially worked with CXCR4 as there is a substantial repertoire of ligands that can be used to asses the functionality of CXCR4, namely; CXCL12 (natural chemokines [isoforms α and γ], conformational antibodies and the HIV-1 envelope glycoprotein). Once the solubilization and immobilization protocol was set-up for CXCR4, we would use the technique to screen for potential HIV-1 entry inhibitors.

Initially, the solubilized coreceptors were used in simple immunoprecipitation experiments, using a solubilization solution that was previously described (Navratilova, Dioszegi et al. 2006) which revealed by western blot that the abovementioned ligands did recognise the solubilized coreceptors. These results were positive and reassuring and thus we proceeded to using the solubilized coreceptors in the SPR experiments. The efficient binding of antibodies (12G5, 4G10 and anti-sulphotyrosine), chemokines (CXCL12α and γ) and viral envelope (X4 gp120) to the solubilized and immobilized CXCR4 strongly suggests that the CXCR4 protein retains its native conformation. 12G5 is a conformationally sensitive antibody that was chosen to asses whether or not the solubilized CXCR4 was correctly folded. The monoclonal conformationally sensitive antibody 12G5

binds a complex epitope, influenced by the integrity of the second extracellular loop and the disulphide bond between cysteine 28 and 274 (Carnec, Quan et al. 2005). The first part of this work (Chapter 5) is a study that was borne from using CXCL12 as a ligand to bind the solubilized and immobilized CXCR4 in order to confirm its functional integrity. CXCL12 is the natural ligand for CXCR4 and interacts with CXCR4 through binding of the N terminus and the second and third extracellular loops (Juarez, Bendall et al. 2004; Gozansky, Louis et al. 2005).

The SPR signal measured by the optical biosensor is sufficiently sensitive to detect low molecular mass compounds (such as chemokines) with a relatively low Rmax. Fitting of the CXCL12α binding curves using a 1:1 langmuir interaction model returned on rates (k_{on} or k_a) of $2.58 \times 10^6 \pm 5.9 \times 10^5$ $M^{-1}s^{-1}$ and off rates (k_{off} or k_d) of $3.36 \times 10^{-2} \pm 5.9 \times 10^{-3}$ s^{-1}. We were able to determine the affinity of CXCL12α for CXCR4 giving rise to a K_D of 13 ± 1.6 nM which is consistent with previously published assays; 3.6 ± 1.6 nM, 4.7 ± 1.6nM and between 1.2 – 27 nM for a similar SPR assay (Crump, Gong et al. 1997; Di Salvo, Koch et al. 2000; Navratilova, Dioszegi et al. 2006). The affinity was also in the same range (3.29 ± 0.52 nM) as an assay using ^{125}I-CXCL12α binding to recombinant CXCR4 (Zhou and Tai 1999). For CXCL12γ, preliminary analysis of the curves showed that the association and dissociation rate constants were dominated by mass transfer (the rate at which the surface binds the analyte exceeds the rate at which the analyte can be delivered to the surface) and thus the binding curves were fitted to a 1:1 langmuir binding model that compensated for mass transport. The association rate constant for CXCL12γ was calculated as $k_{on} = 1.05 \times 10^7 \pm 1.2 \times 10^7$ $M^{-1}s^{-1}$ and dissociation rate constant was calculated as $k_{off} = 5.6 \times 10^{-3} \pm 5.3 \times 10^{-3}$ s^{-1}. As for CXCL12α, a concentration-dependent binding response was observed for CXCL12γ and we calculated an affinity K_D of 0.7 ± 0.3 nM. Interestingly, CXCL12α displayed a lower association rate constant for CXCR4 and the dissociation rate constant for CXCL12γ is slower that for CXCL12α. Here, the CXCL12γ isoform displayed a higher affinity for the immobilized CXCR4 and this meant that CXCL12γ binds stronger to CXCR4 than CXCL12α. Since, the only difference between the CXCL12α and CXCL12γ isoforms is the C-terminal extension, these results suggest that it is this non-structured basic C-terminal extension that is responsible for the augmented affinity that CXCL12γ has for CXCR4.

The mutant M1 is the CXCL12γ chemokine, however, 9 basic amino acids have been mutated in its C-terminal, thus it has an overall less positive charge than that of the CXCL12γ. However M1 still posseses 9 positive charges in its C-terminal domain, thus is more positively charged than CXCL12α. The M1 mutant was an intermediate between the two and interestingly, M1 displayed a binding profile to CXCR4 much like that of CXCL12α. M1 did not stay bound to CXCR4; it dissociated from CXCR4 as did CXCL12α. However its RMax is greater than that of CXCL12α, in the same order of magnitude as that of CXCL12γ. Thus, the fact that CXCL12γ has a higher affinity for CXCR4 and that it does not dissociate from the coreceptor is linked to the high number of basic residues found in the C-Terminal.

Since from our data, the high affinity displayed by CXCL12γ for CXCR4 is due to the basic C-terminal, one should be able to complete the binding to CXCR4

through the presence of anionic GAGs, which have been shown to bind the C-Terminal of CXCL12γ (Laguri, Sadir et al. 2007). Hence, we studied the effect of soluble GAG chains on the interaction between CXCL12α, M1 and CXCL12γ with CXCR4. A 12mer HP oligomer (1μg/ml) did not have any effect on the binding of either CXCL12α or M1 to CXCR4, shown either by SPR or by flow cell cytometry. However, the same size and concentration of oligosaccharide had a drastic effect on CXCL12γ binding to CXCR4. With both techniques (SPR and FACS analysis), the oligosaccharide significantly diminished the binding of CXCL12γ to its coreceptor. This effect was most likely due to a competition between the anionic oligosaccharide and the coreceptor with the basic C-Terminal of CXCL12γ. Again, this effect was not seen in M1, meaning that the competitive binding is taking place in the C-terminal of CXCL12γ. As expected, the M1 binding to CXCR4 was not affected by the presence of 12mer HP oligosaccharides, thus confirming that the strong affinity that CXCL12γ has for CXCR4 is due to its elongated C-terminal. We hypothesised that the basic elongated C-Terminal tail of CXCL12γ may bind to the anionic N-Terminal of CXCR4 which posseses up to three tyrosine residues which can each be sulphated, contributing to the high affinity seen between CXCL12γ and CXCR4.

Tyrosine sulphation occurs in about 1% of the total protein content of a cell and it is mediated post-translationally by tyrosine sulphotransferases in the *trans*-golgi network (Huttner 1988). Sulphated tyrosines have been detected in several chemokine receptors (CCR2B, CCR5 and CX3CR1) and is important for the interactions with their respective ligands (Preobrazhensky, Dragan et al. 2000; Farzan, Babcock et al. 2002; Fong, Alam et al. 2002). Site-directed mutagenesis studies have shown that Tyr26 is sulphated in CCR2B, the monocyte chemotactic protein-1 (MCP-1) receptor and the lack of this sulphation inhibits the biological function of the receptor (Preobrazhensky, Dragan et al. 2000). Interestingly, the addition of heparin does not inhibit chemotaxis, as the heparin binds at the basic C-terminal domain of MCP-1, which is opposite to the site of receptor binding. Preobrazhensky *et al.,* propose a mechanism whereby MCP-1 may be bound to cell-surface GAGs and the N-terminal of CCR2B "grabs" the tethered MCP-1 in order to favour the chemokine binding to the receptor (Preobrazhensky, Dragan et al. 2000). Another example of sulphated tyrosines found in receptors is that of the N-terminal of CCR5 which contributes to the binding of macrophage inflammatory protein -1α, -1β and Regulated on Activation Normal T cell Expressed (RANTES) in leukocyte chemotaxis and activation (Bannert, Craig et al. 2001; Farzan, Babcock et al. 2002) and gp120-CD4 complex binding (Farzan, Mirzabekov et al. 1999). In addition to the sulphated tyrosine, sialylated *O*-glycans within the N-terminal of CCR5 exert little effect on HIV-1 binding, however, are critical for MIP-1α and -1β high affinity binding (Farzan, Choe et al. 1998; Farzan, Mirzabekov et al. 1999; Bannert, Craig et al. 2001; Wang, Babcock et al. 2004).

Considering the complementarity in surface charge of the sulphates in the CXCR4 N-terminal and the highly basic CXCL12γ C-terminal, it was logical to propose that the high affinity that is observed between CXCL12γ and CXCR4 is due to the ionic interaction between the basic amino acids in the C-terminal of CXCL12γ with the sulphated tyrosine residues present in the N-terminal of CXCR4. Thus

we analysed the binding of CXCL12γ on the immobilized CXCR4 surface and subsequent injection of the anti-sulphotyrosine antibody (which recognises the sulphotyrosines in the N-terminal of CXCR4). Once the CXCL12γ was already bound, recognition of the coreceptor by the antisulphotyrosine antibody was blocked. This demonstrated that these two binding events are mutually exclusive. To rule out the effect of steric hindrance preventing the anti-sulphotyrosine antibody from binding once CXCL12γ has already bound, we performed the same experiment with 4G10 (an antibody raised against a non-sulphated N-terminal CXCR4 peptide). Here, both the 4G10 and the CXCL12γ were able to bind the immobilized CXCR4 simultaneously.

CXCR4 is modified by a chondroitin sulphate at Ser18 in some cell lines, however, neither HIV-1 entry nor CXCL12α binding is affected by the loss of this glycosaminoglycan (Farzan, Babcock et al. 2002). In addition to this modification, Asn11 is post-translationally modified by N-linked glycosylation and this alteration is important for high affinity CXCL12α binding and when mutated permits infection of R5 HIV-1 strains and enhances infection by X4 strains (Zhou and Tai 1999; Chabot, Chen et al. 2000; Wang, Babcock et al. 2004). The significance of the N-linked glycan was not investigated here due to the complexity of the experiment. The glycan at Asn 11 is important, however, not essential for binding and we report biochemical and structural data on the significance of the sulphated tyrosines and their roles played in CXCL12γ chemokine binding. Further studies will need to scrutinise the significance of N-linked glycans in CXCL12γ binding to CXCR4.

In 2006, Veldkamp *et al.,* showed that a dynamically disordered CXCR4 peptide (first 38 residues), enzymatically modified to contain one sulphotyrosine residue at position 21, bound the CXCL12α dimer with a low μM affinity and formed a salt bridge with Arg47 on the chemokine (Veldkamp, Seibert et al. 2006). We sought to test the hypothesis that the C-terminal of CXCL12γ binds to the sulphotyrosines in the N-terminal of CXCR4. To this end, structural and cell-based approaches were employed. In our NMR experiment, we obtained two synthetic peptides (produced by collaborators at the Institut Pasteur) which consisted of the N-terminal peptide (29 residues long) of CXCR4. One of the peptides possessed three sulphated tyrosines (7, 12 and 21) and the other peptide contained tyrosines that were not sulphated. For each experiment, ^{15}N labelled CXCL12γ was titrated with incremental additions of either sulphated or non-sulphated N-terminal peptide to identify the residues that were involved in sulphated peptide binding.

We assigned ^{15}N-^{1}H amide chemical shifts of ^{15}N labelled CXCL12γ using standard NMR methods and mapped these onto the CXCL12γ sequence. When the ^{15}N labelled CXCL12γ was titrated with incremental additions of the non-sulphated peptide, the core domain (CXCL12γ $_{1-68}$) was affected, however the C-Terminal of CXCL12γ was not at all affected. A similar result was seen with Veldkamp *et al.,* where the N-terminal of the CXCR4 interacted with CXCL12α $_{1-68}$ as shown in a similar experiment which produced almost identical chemical shifts for the N-terminal folded part of the chemokine (Veldkamp, Seibert et al. 2006; Veldkamp, Seibert et al. 2008). Veldkamp and collegues also showed that even with one sulphated tyrosine on the CXCR4 N-Terminal peptide, no change

in the chemical shifts of CXCL12α $_{1-68}$ were identified. Therefore, they concluded that the sulphates are not significantly involved in the binding of CXCL12α $_{1-68}$.

However, when the ^{15}N labelled CXCL12γ was titrated with incremental additions of the sulphated peptide, the same result was seen for the 'CXCL12α-like' part of CXCL12γ (CXCL12γ $_{1-68}$), thus confirming the work which Veldkamp and collegues performed, and the C-Terminal was also modified. This NMR experiment revealed compelling data that the presence of the sulphates, enables the binding of the CXCR4 N-Terminal peptide to the C-Terminal of CXCL12γ. Thus the higher affinity observed between CXCL12γ and CXCR4 is due to the C-terminus of the chemokine. This was apparent as the C-terminal of the CXCL12γ displayed stronger chemical shifts in the presence of the sulphated peptide as opposed to the non-sulphated peptide. Due to the repetitive sequence and highly unstructured and disordered nature of the C-terminal of CXCL12γ, specific residue information is not available for most residues in this domain. However, a large group of amine peaks in the C-terminal region of the chemokine significantly shift upon addition of the sulphated peptide, confirming that the many basic amino acids in the C-terminal of CXCL12γ considerably contribute to the binding of the sulphated tyrosines in the N-terminal of CXCR4.

The following CXCL12γ residues showed large shift perturbations and thus were shown to interact with the peptide which includes residues from the N-loop; (F13), β1(K24,H25), β2(39-42), β3(48-50) and the α-helix (W57, Y61, L62). Similar data was shown by Veldkamp *et al.,* and the chemokine N-terminus does not participate in the interaction with the N-terminal of CXCR4, which is consistent with the "two site" binding model of CXCL12 for CXCR4 (Crump, Gong et al. 1997; Veldkamp, Seibert et al. 2006).

We have attempted to show, with ELISA based-assays, that the affinity between the CXCL12γ and the CXCR4 N-terminal sulphated peptide was slightly augmented as compared to that for that of CXCL12γ and the CXCR4 non-sulphated peptide. However, the bulk of the interaction between CXCL12γ and the N-terminal of CXCR4 occurs between the N-terminal of the chemokine and not the C-terminal and thus results from these assays are not 100% conclusive.

In chemotaxis experiments, CXCL12γ has a much weaker effect through cell-surface CXCR4 as compared to CXCL12α (Rueda, Balabanian et al. 2008). The reason for this has never been well understood. Only at high concentrations (100nM and higher) of CXCL12γ is a significant signalling effect seen through CXCR4, which equals that of CXCL12α seen at only 1nM. We hypothesized that due to the high affinity between CXCL12γ and the N-terminal of CXCR4, this chemokine is 'tethered' to the N-terminal of the coreceptor and is spatially too far from the activation site and is thus a weaker signalling chemokine. In accordance with the hypothesis that the higher affinity that CXCL12γ has for CXCR4 is due to the interaction of the basic amino acids in the C-terminal of CXCL12γ with the sulphated tyrosines in the N-terminal of CXCR4 – ERK 1/2 activation experiments with the chemokines (CXCL12α, CXCL12γ and M1) in the presence and absence of oligosaccharides were performed.

Our aim was to 'detach' tightly bound CXCL12γ from the N-terminal of CXCR4 so that the chemokine may move closer towards the activation site (which was

previously spatially too far) between the extracellular loops and activate the coreceptor. Here, CEM cells were used as they have very little/ no GAGs expressed on their surface and thus cell-surface GAGs will not obscure results obtained when soluble GAGs are added to the system. When comparing ERK 1/2 activation by the three different chemokines (CXCL12α, M1 and CXCL12γ), the following concentration range of chemokines was used to compare ERK 1/2 activation, 0.5, 5, 50 and 200nM. Since CXCL12γ signals less stongly than CXCL12α (data not shown), when comparing the effect of cell-free GAGs on chemokine induced ERK 1/2 activation, 50nM of CXCL12γ was used to detect an equivalent ERK 1/2 activation detected with 5nM of CXCL12α. We have shown that when in the presence of 10 μg/ml heparin, CXCL12γ induces a 4X stronger activation of ERK 1/2, whereas there is no difference in ERK 1/2 activation for CXCL12α and M1 in the presence of heparin. I.e. the strength of the ERK 1/2 activation signal seen at 50nM together with the presence of heparin is equivalent to that seen at 200nM of CXCL12γ in the absence of the oligosaccharide. This supports our hypothesis that CXCL12γ signals less compared to CXCL12α due to its strong interaction with the sulphotyrosines in the N-terminal of CXCR4. In our model, when in the presence of short sulphated oligosaccharides, the CXCL12γ-N-Terminal CXCR4 interaction is destabilized permitting the chemokine to detach and access the activation site within the extracellular loops of CXCR4, thus soluble heprain could enhance the CXCL12γ signalisation (Figure 5.14 in Chapter 5). Other hypotheses could be proposed such as; the soluble heparin is able to stabilize the disordered C-Terminal of CXCL12γ, thus allowing it to signalise more efficiently and leading to less non-specific binding occuring. Further studies need to investigate whether or not CXCL12γ dimerises in the presence of GAGs, as does CXCL12α, or would the basic C-Terminal of CXCL12γ prevent dimerisation?

We propose a binding model for CXCL12γ and CXCR4 in the presence of cell-associated or cell-free GAGs. When CXCL12γ is bound to the N-terminal of CXCR4, spatially it is restricted from accessing the activation site within the extracellular loops of CXCR4. However, in the proximal presence of either a cell-associated or cell-free GAG, the electrostatic interaction between the C-terminal of the CXCL12γ and the N-terminal of the CXCR4 is disrupted by the presence of the GAG and thus the CXCL12γ is 'released' and can access the activation site within the CXCR4, allowing signalling. This mechanism only occurs for the CXCL12γ isoform, as seen in the scheme in Chapter 5 Figure 5.14; the CXCL12α is not tethered by the N-terminal of the CXCR4 and can easily access the activation site within the extracellular loops of CXCR4.

Until now, very little has been known about the signalling dynamics of CXCL12γ through CXCR4 and the involvement of GAGs in this process. We have started to decrypt this process, however, further cell-based assays should be performed to confirm the effect of GAGs in CXCL12γ-CXCR4 signalling. For example, chemotaxis analysis and calcium mobilization experiments should be performed for the CXCL12γ isoform. Recently, monomeric and dimeric CXCL12α has been shown to inhibit metastasis through different mechanisms depending on their interaction with CXCR4 (Drury, Ziarek et al. 2011). This is partly explained by the different interaction that takes place between the tyrosine sulphated N-

Terminal of CXCR4 and the CXCL12α monomer and CXCL12α dimer which as shown by NMR, binds the preferential monomer of CXCL12α differently from how it binds the constitutive dimer (Drury, Ziarek et al. 2011). However, these results are highly controversial and need to be demonstrated with non-mutant chemokines. It could be an interesting assay to perform the same experiment but with CXCL12γ to see if a similar phenomenon occurs. This may shed light on the differences in signalisation between CXCL12α and CXCL12γ.

CXCL12 is known to participate in a plethora of both homeostatic and pathological processes concerning the immune system, however the mechanisms of regulation of these two opposing forces are unclear. When comparing the functions of CXCL12α and CXCL12γ, it is apparent that due to the difference in structure, these two chemokine isoforms are likely to have diverse functions. Based on numerous studies, CXCL12α binds readily to cell-surface glycosaminoglycans (Sadir, Baleux et al. 2001; Lortat-Jacob, Grosdidier et al. 2002) as well as the sulphated N-terminal of CXCR4 (Veldkamp, Seibert et al. 2006) as does CXCL12γ, however the latter with a 10x higher affinity. CXCL12α will bind to cells expressing a significant quantities of sulphated GAGs (Santiago, Izquierdo et al. 2011), and the degree of binding will be directly correlated with the degree of sulphation of the GAGs. Depending on the cell type, developmental stage and pathophysiological state, the GAG expression profile may vary and this may play a role in the regulation of CXCL12α signalling during development and disease states. Conversely, CXCL12γ has such a a high affinity for GAGs that it may bind to immune cells which express any range of oligosaccharides; from a slight/low expression to a large level of expression and sulphation. Thus the repertoire of immune cells to which these two isoforms will bind and recruit immune cells, might be different. In addition to the different target cells to which the chemokines bind, the kinetics of signalling and motility between these two isoforms is vastly different; CXCL12α has a half life of being bound to the cell surface and its receptor is much shorter than that of CXCL12γ due to their different affinities for their anionic binding partners; this might lead to drastic differences in the extent of signalling.

We hypothesize that the CXCL12γ is expressed and secreted for highly concentrated, local and small haptotactic gradients for sequestration of immune cells over a much more prolonged period of time as opposed to the larger spread-out gradients that are created with CXCL12α which will comparatively be shorter lived. Further studies will be needed to determine if the differential expression of GAGs *in vivo* correlates with a differential retention and downstream signalling effects of either chemokine isoform.

Another hypothesis for the functional diversity of CXCL12γ is that of proteolytic degradation. Since CXCL12γ displays a lower activation frequency of CXCR4 due to its C-Terminal sequence, a level of regulation of CXCL12γ signalling could be through the digestion of the C-terminal. This could occur via proteases including matrix metalloproteinases or serine proteases which could remove the C-terminal of CXCL12γ. This is highly speculative and would need to be thoroughly investigated, but it could be a level of CXCL12γ signalling regulation.

As can be seen, the biological relevance of the chemokine-GAG interaction and chemokine-CXCR4 interaction has clearly been described. However, this complex process has still much to be discovered; the role played by GAGs is particularly intricate as GAGs are structurally hypervariable and this structural variability may have specific functional significance (depending on the level of sulphation, length of GAG chain etc). Many questions are raised such as, for a specific GAG structure, which chemokine ligand is bound, what is the strength and duration of this interaction, what its state of oligomerisation and while bound does it have an ability to signal efficiently and how is this all regulated? Numerous inhibitors have been developed to interfere with these interactions, whether they are chemokines that are mutated to be coreceptor or GAG binding antagonists or whether GAG mimicking peptides or oligosaccharides have been used as chemokine decoys. Thus, while there has been huge progress made in the search for anti-inflammatory molecules and anti-cancer drugs, further understanding of the 'chemokine interactome' will improve existing therapeutic approaches and possibly create new ones.

It is known that as a prerequisite to an array of cellular processes and regulatory events, proteins bind to GAGs. In particular, the chemokine system is intimately associated with GAGs as a means to ensure their correct positioning and transport within tissues (Colditz, Schneider et al. 2007; Rot 2010). CXCL12γ's disordered C-terminal tail contributes to the unprecedented high affinity for GAGs and forms a stable complex. An equivalent high affinity occurs with CXCL12γ and CXCR4. The C-terminal tails on CXCL12γ arises from alternative splicing of the *Cxcl12* gene and this has been thought of as natures' way to generate functional diversity without structural modification nor the appearance of separate proteins (Laguri, Sadir et al. 2007).

9.2.2 The Entry Inhibitor Study

In order to target HIV-1 entry, a successful inhibitor will have to not only block CD4 binding but also block gp120 from binding to its cognate coreceptor (ideally both CCR5 and CXCR4). This is an incredibly challenging task as the HIV-1 glycoprotein surfaces that are involved in entry are well hidden by a glycosylated shield as well as a hidden cryptic CD4i.

Since the discovery of HIV-1 thirty years ago, many different drugs that target the virus have been discovered and approved for treatment in patients. Recently, a new class of molecules has attracted a lot of attention; the entry inhibitors.

As a result of strong positive selection, the occurrence of the 32 base pair deletion (*CCR5 Δ32* allele) in certain individuals results in the altered and reduced expression of CCR5 and thus confers natural immunity to HIV-1 without any negative effects on health (Stephens, Reich et al. 1998). This fact has sparked a lot of interest in developing CCR5 ligands with antiviral properties, such as chemically modified chemokines (Mack, Luckow et al. 1998; Pastore, Picchio et al. 2003), monoclonal antibodies (Trkola, Ketas et al. 2001; Safarian, Carnec et al. 2006) and low molecular weight non-peptidic compounds.

The latter class of entry inhibitors includes Maraviroc (UK-427857, marketed as Selzentry or Celsentry) which has recently been approved in 2007 for treatment of

patients harboring *only* CCR5 utilizing viruses (Dorr, Westby et al. 2005) as it is a molecule that binds to the host CCR5 coreceptor. Maraviroc is also used to prevent HIV-1 transmission. Some promising data was shown by PRO 542 which is the tetravalent CD4-immunoglobulin containing D1 and D2 domains of CD4, however, this compound is no longer being developed. Ibalizumab (TNX-355) is a monoclonal antibody that binds the second domain of CD4 and has also shown promising results. Other CCR5 antagonists have been discontinued (Aplaviroc, INCB009471) or are entering phase III trials (Vicriviroc [SCH417692 or SCH-D]) (McNicholas, Wei et al. 2011), however, there is no existing CXCR4 antagonist. This is not surprising as there are no known naturally occurring mutations leading to the absence of CXCR4 and its expression is essential to the development and immunosurveillance of our organism and blocking this receptor can lead to grave complications. Thus due to toxicity effects, the development of AMD3100 a CXCR4 antagonist, was put on hold. Lastly, a fusion inhibitor that has been approved by the FDA for clinical use, Enfuvirtide (T-20), prevents the association of HR1 and HR2 of gp41. Enfuvirtide is administered to treatment-experienced patients with resistant viruses, however, usage is limited to short term use due to pain at the injection site and resistance develops relatively quickly (Wild, Shugars et al. 1994; Lalezari, Henry et al. 2003; Lazzarin, Clotet et al. 2003).

9.2.2.1 mCD4-HS_{12} and mCD4-$P3YS0_3$

There is a great need for a novel entry inhibitor that binds to the virus which has low toxicity and inhibits both CCR5 and CXCR4-tropic HIV-1 variants. For this purpose we aimed to increase the specificity and affinity of the prototype bivalent entry inhibitor molecule, mCD4-HS_{12} (Baleux, Loureiro-Morais et al. 2009).

It is known that in order to target the vulnerable coreceptor binding pocket (CD4i epitope), a molecule with anionic properties would be required to complementarily dock into the CD4i epitope comprising a number of basic residues. Several studies have shown that anionic molecules are able to inhibit mAb 17b binding with IC_{50} in the 1-100μM range (Cormier, Persuh et al. 2000; Farzan, Vasilieva et al. 2000; Cohen, Forzan et al. 2008; Crublet, Andrieu et al. 2008; Brower, Schon et al. 2009; Dervillez, Klaukien et al. 2010; Acharya, Dogo-Isonagie et al. 2011; Kwong, Dorfman et al. 2011; Seitz, Rusert et al. 2011). Crublet *et al.,* showed that HS belongs to this group of anionic CD4i targeting molecules and when a 12mer was conjugated to a mini CD4, this bivalent molecule had profound antiviral properties (Baleux, Loureiro-Morais et al. 2009). Due to the extremely complex and heterogeneous nature of HS (Esko and Lindahl 2001), based on the 48 different disaccharide combinations, a 12 mer library ($48^6 = 10^{10}$) would be inconceivable to synthesize for structure-function studies. In addition, assembling the 12mer used in the prototype took 1 year to synthesize and thus a larger library of differently sulphated 12mers would be impractical to produce.

Thus, the glyco-portion of the prototype needed to be improved/optimized and there were two ways to do this: one approach would have been to fractionate a library of natural 12mer heparan sulphate oligosaccharides by means of ion-exchange chromatography under the influence of an increasing salt gradient, purify and test each fraction for its ability to inhibit gp120-CD4 binding to the

coreceptor and then purify the most active fraction in large quantities. This approach would be tedious and also not amenable to structure-function analysis as this purification technique is far from accurate and a homogeneous solution of one type of 12mer structure would not be possible to obtain. Even if an oligosaccharide 12mer with high binding affinity to gp120 was identified, sequencing of this 12mer to identify its exact disaccharidic structure would also be very tedious.

The second approach was the design of HS-mimetic peptides containing negatively charged amino acids that mimic the HS moieties and this would simplify the process twofold; the peptides are straightforward to synthesize and a homogeneous known peptide sequence would be amenable to sequence-activity investigation. To mimic the functional hydroxyl, carboxyl and sulphate groups found on HS moieties, peptides containing serine, asparagine and tyrosines (either sulphated or non-sulphated) were produced. The $S(XDXS)_3$ sequence was used where X represents any amino acid and five peptides were synthesized; P3Y (Where X = non-sulphated tyrosine), $P3YSO_3$ (where X = sulphotyrosine), P3Asu (where X = aminosuberic acid), P3pF (where X = p-carboxymethyl phenylalanine) and E13 (where the entire length is glutamic acid – a non-specific polyanion). The sulphotyrosine-containing peptides ($P3YSO_3$) were shown to block mAb 17b with an IC_{50} of 3μM, which compares well to the previously published molecules, additionally, when conjugated to mCD4, $P3YSO_3$ interacted with both R5 and X4 gp120, however HS_{12} bound the X4gp120 with preference.

When $P3YSO_3$ was covalently coupled to mCD4, a synergistic effect was seen and low nM amounts of mCD4-$P3YSO_3$ were needed to prevent gp120-CD4 from binding to mAb 17b. Since mAb 17b is only a rough surrogate coreceptor, the inhibitory capacity of mCD4-$P3YSO_3$ needed to be tested on functional solubilized CCR5 and CXCR4 immobilized on biacore surfaces.

Using the GPCR solubilization protocol that had been set-up and optimized in Chapter 5, both CCR5 and CXCR4 were immobilized on the chip and interactions between gp120, in the presence and absence of mCD4 were monitored. Binding of gp120 to its coreceptor was CD4 dependent, however, there was binding between the coreceptor and the gp120 in the absence of CD4. This can be explained by a percentage of the envelopes being 'CD4-independent' or 'pre-triggered'. This phenomenon has been documented by different techniques; previously, CCR5-containing proteoliposomes have been shown to bind R5 gp120 in the absence of soluble CD4, however, a stronger binding interaction is seen in the presence of sCD4 (Mirzabekov, Kontos et al. 2000; Babcock, Mirzabekov et al. 2001). To the contrary, Babcock *et al.,* did not detect CD4 independence for CXCR4 utilizing envelopes.

Babcock *et al.*, calculated the affinity of HXBc2 gp120 for CXCR4 to be ~200nM (Babcock, Mirzabekov et al. 2001), nearly 100-fold lower than that of R5 HIV-1 estimated by Wu *et al.*, (Wu, Gerard et al. 1996). A similar affinity was calculated for gp120 binding to CXCR4 (~500nM) for virion-like particles incorporating CXCR4 (Hoffman, Canziani et al. 2000). Binding of the gp120-CD4 complexes to the solubilized GPCRs was also dose-dependent. We obtained an affinity of 154 ± 68 nM for the X4 gp120/CD4 – CXCR4 interaction which is comparable to that

obtained in a cellular system (Doranz, Baik et al. 1999; Babcock, Mirzabekov et al. 2001) and 11.5 ± 2.9 nM for the gp120/CD4-CCR5 interaction which are in agreement with that found for a similar technique (Navratilova, Sodroski et al. 2005). The preference for HIV-1 isolates to infect CCR5-expressing host cells in the early stages of infection could be explained by this apparent 'lower' affinity for CXCR4. As our assay results are consistent with previously published data for the affinities of gp120-CD4 complexes for their respective coreceptors, our system was clearly functional and we proceeded to investigate the inhibitory capacity of the above mentioned synthetic peptides.

Out of the 5 bivalent peptides that were screened on the GPCR surfaces, mCD4-P3YSO$_3$ was the only peptide that fully inhibited the gp120 binding to both CCR5 and CXCR4 coreceptors with a 1:1 stoichiometry. One of the criticisms of many new potential entry inhibitors is how the molecule will inhibit *both* CCR5 and CXCR4-utilizing viruses. This will be achieved by mCD4-P3YSO$_3$ through its bivalent nature; it is known that the V3 loop which comprises part of the coreceptor binding domain (Dragic 2001) has a more basic charge in X4 envelopes as compared to that of R5 envelopes (Moulard, Lortat-Jacob et al. 2000). Thus in order for the mCD4-P3YSO$_3$ peptide to target a wide range of envelopes with a range of different overall charges in their V3 loop, we propose sulphated peptides that have moderate levels of sulphation and that the specificity of the binding interaction will be attributed to the mCD4 moiety.

Interestingly, the other bivalent HIV-1 entry inhibitor peptides (mCD4-PSY, mCD4-P3Asu, mCD4-P3pF and mCD4-E13) all displayed varying degrees of antiviral activities in the PBMC infectivity assay against the LAI (X4 tropic) HIV-1 strain. However, they were all ineffective against the Ba-L (R5 tropic) strain. This is understandable as the other peptides all contained negatively charged amino acids (carboxyl groups) which most likely bound to the basic V3 loop of the gp120, which carries a particularly higher positive charge in X4 tropic HIV-1 strains (De Jong, De Ronde et al. 1992). Thus, these other peptides are not redundant, they can also be envisaged as antiviral therapies that are administered as a salvage therapy in addition to compounds against R5-tropic viruses (eg Maraviroc) for patients who have CXCR4-utilizing or dual-tropic HIV-1 strains.

By replacing the glyco-moiety of mCD4-HS$_{12}$ with a tyrosine sulphated peptide, one can begin to have a rough idea of the quantity and position of sulphated residues that are minimally required to bind to a certain epitope on a protein. Not only is this sulphated peptide of value as a new generation HIV-1 entry inhibitor but it also opens up a whole new strategy for determining the structure-function relationship between sulphated molecules and target proteins.

This new molecule, mCD4-P3YSO$_3$, has an IC$_{50}$ as low as 1 nM and thus deserves to be tested in macaques against the SHIV model of HIV for its efficacy in an animal model. Many questions are raised when novel therapeutic drugs pass in the pipeline from basic research to animal models and finally to clinical trials; such questions concern the molecule's distribution in the host, its stability, immunogenicity, ease of production and what if resistance develops in the virus against the drug. Depending on whether mCD4-P3YSO$_3$ is used as a treatment or whether it is used as a prevention strategy, this will affect the molecules'

distribution. As a preventative approach, mCD4-P3YSO$_3$ can be incorporated into a mucosal gel that will be applied to the vaginal and anal mucosa and thus be used as a microbicide. In this case, the distribution of the drug will be local and concentrated at the genital mucosal tract – the first site of entry of the virus during sexual transmission of the virus. However, if mCD4-P3YSO$_3$ is used as a treatment, it will need to be administered intravenously or subcutaneously at regular intervals to counteract the possible degradation or hydrolysis of the molecule by host proteases. Thus the drug's distribution within the blood will depend upon the regularity of the injections. Since the mCD4-P3YSO$_3$ is devoid of toxicity up to 1 μM in cell culture, this bodes well for toxicity tests in the animal model.

In terms of mCD4-P3YSO$_3$ stability, one approach that could be used to protect the anionic peptide part of the bivalent molecule from host protease hydrolysis, will be to use the stereoisomers of each amino acid. *D* amino acids are found in nature, however, *L* amino acids are mostly used to form proteins in organisms (*D* amino acids are found in frogs skin, in particular). Since the target recognition for the anionic part of the bivalent molecule is not strictly reliant on the structure (as is the case for the mCD4 part), *D* isomers can be used as they will still serve their function by placing a negative charge at a certain position, regardless of their chirality. Thus, *D* isomers will not be recognized by the host and thus not degraded by the host proteases.

Immunogenicity screening needs to be performed in an animal model to test the ability of mCD4-P$_3$YSO$_3$ to produce an immune response. Due to the fact that the mCD4 moiety is 27 amino acids in length, containing only several amino acids dereived from the host CD4 protein, when conjugated to the 'HS mimetic' moiety (P3YSO$_3$), the final molecule is essentially a short peptide containing sulphotyrosines. The very small size of this molecule reduces the chances for the host to mount an autoimmune reaction against it.

Concerning the production of mCD4-P$_3$YSO$_3$, as mentioned previously, the prototype mCD4-HS$_{12}$ took up to one year to synthesize and the HS$_{12}$ moiety is very unstable. Our second generation bivalent entry inhibitor is thus already much faster to synthesize compared to the prototype and mCD4-P$_3$YSO$_3$ is much more stable compared to mCD4-HS$_{12}$. To further reduce the production time of mCD4-P$_3$YSO$_3$, one could replace the sulphate (SO_4^{2-}) residues on the tyrosines with sulphonate (SO_2O^-) residues, which only differ by one oxygen atom and are technically much easier to add to a peptide chain being synthesized. However, sulphonate containing peptides will need to be screened for their ability to inhibit gp120-CD4 complexes from binding to host coreceptors.

In addition to the usual problems associated with anti-viral drug design (selectivity, oral bioavailability, etc.), development of novel HIV-1 entry inhibitors are hindered by problems associated with rapid evolution of the virus, leading to drug resistance. For example, by blocking only CCR5-utilizing viruses from entry into host cells, the CXCR4-utilizing viruses could become dominant. This is the problem for CCR5 antagonists, which could potentially accelerate the progress of the disease by promoting the evolution of more virulent, CXCR4-dependent variants. Our bivalent entry inhibitor (mCD4-P3YSO$_3$) targets both

CCR5- and CXCR4-utilizing HIV-1 variants and this molecule is unlikely to induce mutations within the envelope to escape binding, as development of mutations in two different parts of the protein simultaneously is probably difficult for the virus. However, if resistance does develop to this inhibitor it could be used in combination with other antiretrovirals or as prophylactic treatment.

Structural information of the mCD4-P3YSO$_3$ interacting with HIV-1 gp120 is critical for understanding the molecular basis of the viral - coreceptor (CCR5 and CXCR4) interactions. This information will shed light on the HIV-1 entry mechanism and aid in developing other specific inhibitors of these interactions. In 2007 Huang *et al.,* and colleagues showed the interaction between the tyrosine sulphated CCR5 N-terminus (NMR structure) and the tyrosine sulphated 412d antibody (X-Ray crystal structure) each in complex with gp120 and CD4 (Huang, Eshleman et al. 2007). Here, we are privileged to see the exact contacts that are formed between the sulphated tyrosine (from the N-terminal of CCR5 and the 412d extended loop) and the gp120 binding pocket. Tyr10 interacts with the gp120 core and forms a salt bridge with Arg327 of gp120 and the Tyr14 packs against the bridging sheet (Huang, Eshleman et al. 2007). The next step for our project is to crystallize the complex of mCD4-P3YSO$_3$ together with gp120 so that the results can be used to determine the exact binding sites between mCD4-P3YSO$_3$ and gp120. From these an even higher affinity inhibitor can be designed containing only the essential sulphotyrosines involved in the binding interaction, which may be less than are found in mCD4-P3YSO$_3$. Eventually a trimeric mCD4-P3YSO$_3$ can be modeled and produced, which will be challenging from a synthetic point of view, but most likely more efficient at blocking viral entry as it will bind an entire envelope trimer instead of an envelope monomer (however, this might be less bioavailable).

This compound shows no toxicity up to 1μM and inhibits entry of CCR5 and CXCR4 utilizing laboratory adapted viruses (LAI [X4] and Ba-L [R5]) as well as primary viral strains from diverse subtypes with an ED$_{50}$ of 1nM. The mCD4-P3YSO$_3$ demonstrated a low nM ED$_{50}$ (0.2 – 1.2 nM) for 5 clinical primary isolates (X4 clade A [92UG029], R5 clade B [SF162], X4R5 clade B [92US723], X4R5 clade B [96USHIPS4], X4 clade B [92HT599]) and it displayed an ED$_{50}$ of 29nM for an X4 tropic clade C virus [98IN017]. This dual tropic antiviral activity is unprecedented for any HIV-1 entry inhibitor. These data further demonstrate the high anti-viral capacity of this molecule. The information provided by this study will be useful for future design of novel anti-viral molecules as therapeutics for HIV-1 infection as well as other therapeutic molecules against pathogens whose activity relies on recognising a host receptor as well as an anionic surface GAG.

PUBLICATIONS AND COMMUNICATIONS

Publications

Accepted

Connell BJ, Françoise Baleux, Yves-Marie Coic, Pascal Clayette, David Bonnaffé and Hugues Lortat-Jacob. 2012. A Synthetic heparan sulphate-mimetic peptide conjugated to a mini CD4 displays very high anti-HIV-1 activity independently of coreceptor usage, *Chemistry & biology* 19:131-139.

In preparation

Connell BJ, Sadir R, Laguri C, Baleux F, and Lortat-Jacob H. (2012) Heparan Sulphate differentially regulates the binding of SDF α and γ to CXCR4.

Connell BJ, INDUS© authors, Lortat-Jacob H. (2012) Cinnamon derived compounds display anti-HIV-1 activity by blocking the heparan sulfate and the coreceptor binding sites of gp120.

Communications

Connell BJ, Baleux F, Hersant Y, Bonnaffé D, and Lortat-Jacob H (2011). A Surface Plasmon Resonance (SPR) Based Screening Assay for the Development of Glyco-Based Anionic Molecules as HIV-1 Entry Inhibitors. Keystone Conference: "HIV Evolution, Genomics and Pathogenesis", March 2011, Whistler, British Columbia, Canada

Connell BJ, Sadir R, Laguri C, Baleux F, and Lortat-Jacob H. (2011) Heparan Sulphate differentially regulates the binding of SDF α and γ to CXCR4. 7th International Conference on Proteoglycans held in conjunction with the Matrix Biology Society of Australia and New Zealand Annual Meeting. 16-20 October 2011, Sydney Australia.

Chemistry & Biology
Article

A Synthetic Heparan Sulfate-Mimetic Peptide Conjugated to a Mini CD4 Displays Very High Anti-HIV-1 Activity Independently of Coreceptor Usage

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SUMMARY

The HIV-1 envelope gp120, which features both the virus receptor (CD4) and coreceptor (CCR5/CXCR4) binding sites, offers multiple sites for therapeutic intervention. However, the latter becomes exposed, thus vulnerable to inhibition, only transiently when the virus has already bound cellular CD4. To pierce this defense mechanism, we engineered a series of heparan sulfate mimicking tridecapeptides and showed that one of them target the gp120 coreceptor binding site with μM affinity. Covalently linked to a CD4-mimetic that binds to gp120 and renders the coreceptor binding domain available to be targeted, the conjugated tridecapeptide now displays nanomolar affinity for its target. Using solubilized coreceptors captured on top of sensorchip we show that it inhibits gp120 binding to both CCR5 and CXCR4 and in peripheral blood mononuclear cells broadly inhibits HIV-1 replication with an IC_{50} of 1 nM.

INTRODUCTION

Although tremendous progress has been made in the development of antiviral drugs to treat human immunodeficiency virus (HIV-1) infection (De Clercq, 2007) and despite the availability of some 25 approved antiretroviral compounds (most of which target HIV-1 enzymes), the virus continues to be a major concern and remains one of the leading causes of death worldwide. The rapid emergence of drug-resistant viral strains, the inability of current therapy to completely eradicate the virus, and the strong adverse side effects associated with their long-term use (Shafer and Schapiro, 2008) compromise treatment in patients benefiting from these therapies and make the development of new therapeutic options of utmost importance (Flexner, 2007). Inhibition of HIV-1 entry, a process based on the sequential interaction of the viral glycoprotein (gp120) with the cell surface CD4 (Klatzmann et al., 1984) and either one of the two chemokine receptors CCR5 or CXCR4 (Alkhatib et al., 1996; Feng et al., 1996), holds particular promise in addressing complications of current therapy and has become a compelling target for controlling viral replication (Tilton and Doms, 2010). The recent approval of maraviroc, a CCR5 antagonist (Dorr et al., 2005; Maeda et al., 2004), has validated entry inhibition as a viable approach. However, to avoid the selection of pre-existing and more pathogenic CXCR4-using HIV-1 (for which no effective antagonistic inhibitors yet exist) maraviroc has been licensed for the treatment of patients infected with viral strains using CCR5 only.

On the virus side, the gp120 constitutes the central element for all interactive events occurring during the pre-entry steps. A wealth of evidence has shown that gp120 binding to CD4 not only permits virus attachment, but also triggers extensive conformational changes of the envelope that fold and/or expose a four-stranded β sheet, known as the CD4-induced (CD4i) domain (Wu et al., 1996). Being critically involved in CCR5/CXCR4 recognition and highly conserved, this domain represents an attractive pharmacological target. Although inhibition of protein-protein interactions is clearly challenging, a striking feature of the CD4i domain is its basic nature (Kwong et al., 1998; Rizzuto et al., 1998) and, not surprisingly, many of this domain's ligands are characteristically acidic. This includes peptides selected by phage display screening (Dervillez et al., 2010), sulfated oligosaccharides from the heparan sulfate (HS) family (Crublet et al., 2008; Vivès et al., 2005), aptamers (Cohen et al., 2008), peptides derived from neutralizing antibodies (Dorfman et al., 2006), compounds issued from in silico screening of molecular libraries (Acharya et al., 2011), or peptides derived from the N-terminal sequence of CCR5 itself that comprise sulfotyrosines importantly contributing to gp120 binding (Cormier et al., 2000; Farzan et al., 2000). The cryptic nature of this CD4i surface prior to CD4 binding, however, limits its accessibility both temporally and spatially and makes it a relatively intractable pharmacological target. In that context, we recently developed a class of compounds, in which a CD4 mimetic peptide (mCD4) was linked to a HS dodecasaccharide (HS_{12}), and showed that mCD4 exposed the gp120 CD4i domain and renders it available to be blocked by the HS_{12} oligosaccharide (Baleux et al., 2009).

Here, to further develop this concept we engineered a series of tridecapeptides that mimic HS, the synthesis of which,

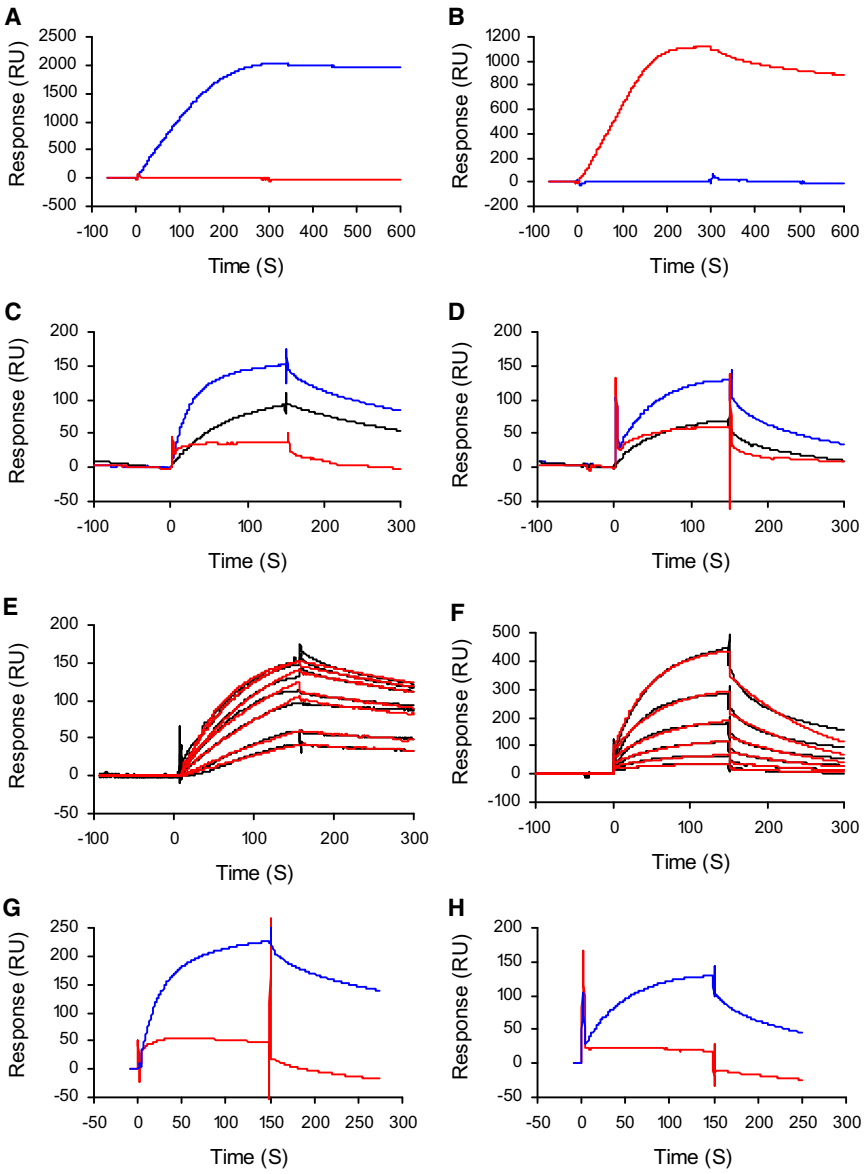


Figure 1. Ligand Binding to CCR5 and CXCR4 Immobilized Sensorchips

Carboxy-terminal C9 tagged CCR5 or CXCR4 were solubilized from Cf2Th cells and captured on top of a mAb 1D4 activated CM4 sensorchip. CCR5 (left) and CXCR4 (right) ligands were injected over the coreceptor surfaces, and the binding responses (in RU) were recorded as a function of time (in S). Binding of 25 nM of mAb 2D7 (blue) and mAb 12G5 (red) to CCR5 (A) and CXCR4 (B). Binding of YU2 gp120 (black), YU2/mCD4 (blue), or YU2/mCD4/maraviroc (red) to CCR5 (C) or MN gp120 (black), MN/mCD4 (blue), or MN/mCD4/AMD3100 (red) to CXCR4 (D). Binding of the equimolar complex of YU2/mCD4 at (from top to bottom) 100, 66, 44, 29, 19, and 12.5 nM to CCR5 (E) or equimolar complex of MN/mCD4 at (from top to bottom) 225, 150, 100, 66, 44, and 29 nM to CXCR4 (F). The black traces correspond to the experimental data, and the red traces correspond to the fitted data using a 1:1 langmuir model. Binding of YU2 or MN gp120 (100 nM) preincubated with 100 nM of mCD4 (blue) or mCD4-HS_{12} (red) to CCR5 (G) or CXCR4 (H).

although amenable to large-scale production, remains extraordinary complex (Dilhas et al., 2008). We then set up a binding assay in which detergent solubilized CCR5 and CXCR4 were both functionally captured on top of sensorchips and used them to show that, conjugated to a mini CD4, a HS mimicking peptide efficiently targets the CD4i domain of gp120 and blocks its interaction with the coreceptors. This compound displays antiviral activity against LAI and Ba-L HIV strains with an IC_{50} as low as 1 nM, two to four orders of magnitude lower than the above-described anionic compounds. To our knowledge, this is the most potent gp120 targeting molecule, with the unique property to simultaneously block two critical and conserved regions of gp120. Importantly it inhibits CCR5 and CXCR4 using viruses equally well, and is also highly active against a number of viral primary clinical isolates. These results should have strong implications for the development of a new anti-HIV-1 therapy.

RESULTS

HIV-1 Coreceptors Immobilization and gp120 Binding

Assessing the ability of molecules to target the coreceptor binding site of gp120 would strongly benefit from a direct coreceptor-gp120 interaction assay. To that end, both HIV-1 coreceptors were solubilized from Cf2Th cells, recombinantly expressing either CCR5 or CXCR4, using a specific cocktail of lipids and detergents that was adapted from that previously described (Navratilova et al., 2005). Solubilized coreceptors, which feature a C-terminal C9 tag (Babcock et al., 2001; Mirzabekov et al., 1999) allowing their oriented capture with the cognate 1D4 antibody, were immobilized on top of a sensorchip to a level of ~4,000 resonance units (RU). To verify whether the coreceptors remained functional, we first investigated their binding capacity with the conformationally sensitive mAb 2D7 for CCR5 (Khurana et al., 2005; Lee et al., 1999) and 12G5 for CXCR4 (Baribaud et al., 2001). As shown in Figures 1A and 1B, injection of these mAbs over the CCR5 and CXCR4 functionalized surfaces gave rise to strong and coreceptor-specific binding signals, indicating both the presence of the coreceptor on the surface and the integrity of the corresponding epitopes.

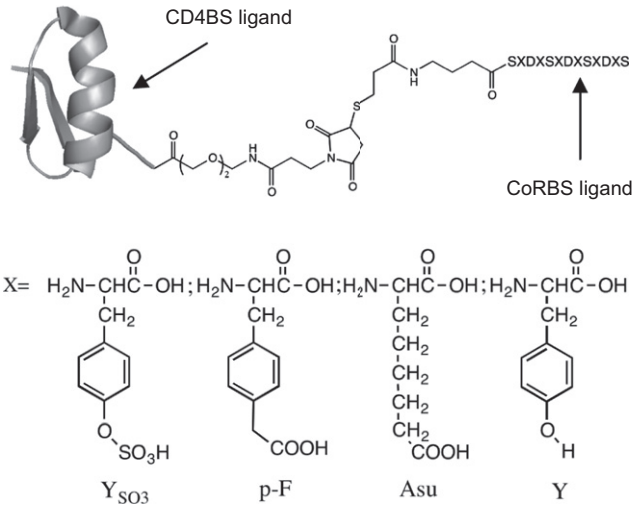
Following this, we analyzed whether the immobilized coreceptors bound gp120, in a CD4-dependent manner. For that purpose, 100 nM of either YU2 or MN (R5 and X4 envelopes respectively), in the absence or presence of mCD4, a CD4 mimetic peptide that was previously found to bind gp120 and induce the conformational change that lead to the folding/exposure of the coreceptor binding site (Baleux et al., 2009),

was injected over the coreceptor surfaces. Both envelopes interacted with their coreceptors, presumably because the CD4i epitope is transiently exposed on the dynamic structure of gp120, as already observed with anti-CD4i antibodies (Thali et al., 1993). The binding responses, however, were significantly enhanced by the presence of mCD4 and efficiently inhibited by 1 μM of maraviroc or AMD3100 (Figures 1C and 1D), two compounds targeting CCR5 and CXCR4 respectively, and having anti-HIV-1 activity (Tilton and Doms, 2010). Next, dose-response experiments were performed with mCD4:gp120 ratios fixed at 1:1 and injected over the immobilized CCR5 or CXCR4 surfaces. Sensorgrams were obtained for both envelopes (Figures 1E and 1F), which evaluations (see Supplemental Experimental Procedures) returned estimated affinities of 11.5 ± 2.9 nM and 154 ± 68 nM for CCR5 and CXCR4, respectively. These values were identical to that reported by a similar technique (Navratilova et al., 2005) or radioligand binding assay with cell membrane-embedded CCR5 (Doranz et al., 1999) as to that reported for CXCR4, using proteoliposome embedded coreceptors and radiolabeled gp120 (Babcock et al., 2001).

We previously reported that the gp120 CD4i epitope can be targeted by HS (Crublet et al., 2008; Vivès et al., 2005), and that a HS dodecasaccharide covalently linked to mCD4 (mCD4-HS_{12}) binds gp120 and blocks its subsequent interaction with mAb 17b (Baleux et al., 2009). mAb 17b belongs to a group known as "anti-CD4i" antibodies, which recognizes a conserved element of gp120, induced by CD4 and partially overlapping the coreceptor binding site (Xiang et al., 2002). We thus made use of the coreceptor binding assay described above to investigate whether mCD4-HS_{12} would also inhibit gp120 binding to CCR5 and CXCR4. As shown in Figures 1G and 1H, both YU2 and MN gp120 in complex with mCD4-HS_{12} featured a strongly reduced ability to recognize CCR5 or CXCR4 compared to that of gp120 in complex with mCD4 alone. This suggests that such molecules could serve as lead compounds for the future development of a new class of entry inhibitors.

Chemical Synthesis of mCD4 Linked HS Mimetic Peptides

HSs are, however, notoriously difficult to synthesize. In addition, their inherent sequence heterogeneity, in terms of sulfation pattern and saccharide composition, would currently make the preparation of a dodecamer series out of reach. Thus, based on the mCD4-HS_{12} template, we tested the hypothesis that the HS moiety could be mimicked by peptides, the chemical synthesis of which is more straightforward, and more easily amenable to sequence-activity relationship investigation. To display the functional hydroxyl, carboxyl, and sulfate groups that characterize HS, peptides comprising Ser, Asp, and Tyr, the latter being possibly sulfated, were considered. This strategy is supported by the observation that a SYDY tetrapeptide binds to the HS binding domain of the vascular endothelial growth factor (Maynard and Hubbell, 2005) and that phage display screenings against the CD4i epitope of gp120 returned sequences enriched in YD motifs (Dervillez et al., 2010). It is also worth noting that a number of antibodies against the gp120 coreceptor binding domains feature sulfotyrosines in their paratope, as does the N terminus of both CCR5 and CXCR4 (Choe et al., 2003).



X	S(XDXS)$_3$ name
YSO_3	P3YSO_3
pF	P3pF
Asu	P3Asu
Y	P3Y

Figure 2. mCD4-S(XDXS)$_3$ Constructs

A miniCD4 was used as a CD4 binding site (CD4BS) ligand and covalently conjugated through an appropriate linker to S(XDXS)$_3$ peptides investigated as potential coreceptor binding site (CoRBS) ligands. S and D are serine and aspartic acid residues respectively and X is either a sulfotyrosine (Y_{SO3}), a p-carboxymethyl phenylalanine (pF) an aminosuberic acid (Asu) or a tyrosine (Y). See also Figures S1 and S2 and Table S1.

Building of a S(XDXS)$_n$ sequence (were X stands for different possible amino acids; see below) using the peptide builder of Hyperchem 5, showed that a 13 amino acid peptide (n = 3), in its extended configuration (φ, ψ, and ω angles set to 180°) would have a length equivalent to the HS 12 mer (data not shown). Thus, a tridecapeptide, alternating OH/COO^- and OH/SO_3^- groups, having the sequence: $SY_{SO3}DY_{SO3}SY_{SO3}DY_{SO3}SY_{SO3}DY_{SO3}S$ (X being in this case a sulfotyrosine; Y_{SO3}) was first synthesized (P3YSO_3). The nonsulfated equivalent (P3Y) was also prepared along with a number of other peptides in which X was replaced by p-carboxymethyl phenylalanine (P3pF) or aminosuberic acid (P3Asu), two residues that have been shown to functionally mimic sulfotyrosine in cholecystokinin type B receptor ligand CCK8 (McCort-Tranchepain et al., 1992) and sulfakinins (Nachman et al., 2005). A tridecaglutamate (displaying 13 carboxylic groups) was also prepared (E13) as a nonspecific poly anionic peptide (Figure 2). In order to maintain an appropriate distance between mCD4 and these peptides, enabling the final molecule to reach both the CD4 and coreceptor binding sites, a γ-aminobutyric acid (γ-Abu) was introduced on their N terminus. These peptides were derivatized with S-acetylthiopropionic acid to allow the coupling to Lys^5 of a maleimide-activated mCD4. All compounds were purified to a level of 95% by RP-HPLC (see Table S1 and Figures S1 and S2 available online), controlled by mass spectrometry and

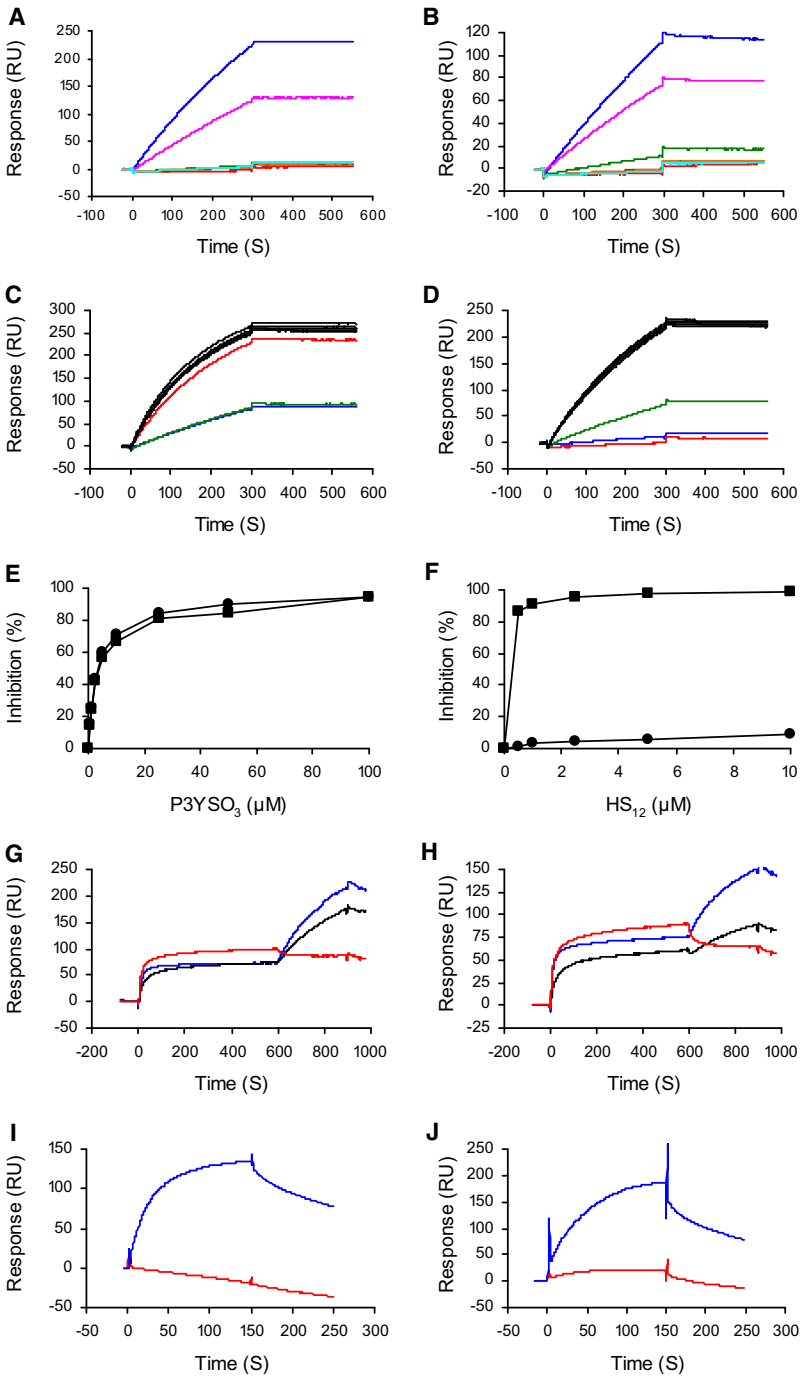


Figure 3. The S(XDXS)$_3$ HS Mimetic Peptides Coupled to mCD4 Inhibit gp120-CD4, gp120-mAb 17b, and gp120-Coreceptor Interactions through Binding to the CD4 and the Coreceptor Binding Sites of gp120

Binding responses measured when YU2 (A) or MN (B) gp120 at 100 nM, either alone (blue) or preincubated with 100 nM of mCD4 (pink), mCD4-P3Y (green), mCD4-E13 (turquoise), mCD4-P3pF (orange), mCD4-P3Asu (brown), or mCD4-P3YSO$_3$ (red) were injected over a CD4 activated surface. YU2-mCD4 (C) or MN-mCD4 (D) complexes (25 nM) were preincubated with 5 μM of HS$_{12}$ (red), P3YS0$_3$ (green), or the other HS mimetic peptides (none, P3Y, E13, P3pF, and P3Asu; all in black) and injected over a mAb 17b activated surface. The blue trace shows the binding of gp120 to mAb 17b in the absence of mCD4. The P3YSO$_3$ peptide (E) or HS$_{12}$ (F) at different concentrations were coincubated with YU2-mCD4 (circle) or MN-mCD4 (square) and injected over a mAb 17b surface. The binding response (mean of triplicate experiment) recorded at the end of the injection phase was plotted versus the concentration of the inhibitors in μM. Overlay of sensorgrams showing the injection of 100 nM of mCD4 (blue), mCD4-P3Y (black) or mCD4-P3YSO$_3$ (red), from 0 to 600 s, over immobilized YU2 (G) or MN (H) gp120, after which 15 μg/ml of mAb 17b was injected from 600 to 900 s. Binding of YU2 or MN gp120 (100 nM) preincubated with 100 nM of mCD4 (blue) or mCD4-P3YSO$_3$ (red) to CCR5 (I) or CXCR4 (J). In all graphs, binding signals were recorded in RU as a function of time (S).

quantified by amino acid analysis as described in the Supplemental Experimental Procedures.

mCD4 Linked HS Mimetic Peptides Inhibit Binding of gp120 to CD4, mAb 17b, and Coreceptors

To verify that peptide conjugation did not prevent the ability of mCD4 to interact with gp120, a competition assay was performed, in which YU2 or MN were incubated with the different mCD4 conjugates and injected over a CD4 functionalized surface. Results showed that the mCD4 conjugates all very efficiently prevent gp120-CD4 interaction, with greater potency than that of unconjugated mCD4 (Figures 3A and 3B). Next, the capacity of the anionic peptides to target the gp120 CD4i epitope was investigated by analyzing their ability to prevent gp120 binding to mAb 17b, in the presence of soluble mCD4. While unliganded gp120 was not (MN) or only poorly (YU2) recognized by mAb 17b (Figures 3C and 3D; blue trace), preincubation with mCD4 strongly promoted binding (black trace). When the gp120-mCD4 complexes were further incubated with 5 μM of the above-described tridecapeptides, strong inhibition was observed for P3YSO$_3$ (green trace). The tridecaglutamate (E13) was devoid of activity, indicating that the anionic character of the peptide is not sufficient to provide binding, as were the unsulfated P3Y or the sequence in which the sulfotyrosine mimetics (pF and Asu) were introduced (Figures 3C and 3D, black traces). HS$_{12}$ (red trace) also fully blocked mAb17b binding to MN-, but not to YU2-gp120. Together, this showed that among the different peptides investigated only the SY$_{SO3}$DY$_{SO3}$ motif competes with mAb 17b to interact with the gp120 CD4i domain. To better quantify the inhibitory activity of this peptide, the same assay was run, with a range of P3YSO$_3$ concentrations, and compared with HS$_{12}$. A similar concentration dependency was observed on both R5 (YU2) and X4 (MN) envelopes, with IC$_{50}$ of 2.9 and 3.1 μM, respectively, indicating that, interestingly, P3YSO$_3$ interacts with gp120 independently

of coreceptor tropism. In contrast, HS_{12} strongly inhibited the interaction between MN and mAb 17b (with a concentration as low as 0.5 μM) but was ineffective toward YU2, at concentrations up to 10 μM (Figures 3E and 3F). Next, to determine the binding mechanism of the mCD4-$S(XDXS)_3$ constructs, X4- and R5-gp120 were immobilized on a sensorchip and first allowed to bind to mCD4, mCD4-P3Y, or mCD4-$P3YSO_3$. The resulting complexes were then probed with mAb 17b, the binding of which being a marker of the coreceptor binding site accessibility. As expected, mCD4 binding to gp120 renders the coreceptor binding site accessible, a point that was also observed, although with a lower efficiency, with mCD4-P3Y. These data indicate that while mCD4-P3Y bound to gp120, the unsulfated peptide did not sufficiently interact with the newly available surface to block mAb 17b recognition. In contrast, when mCD4-$P3YSO_3$ was used instead of mCD4 or mCD4-P3Y, the mAb 17b was no longer able to interact with the complex. Altogether, these data thus support the view that mCD4 first binds to gp120 and exposes the coreceptor binding site, with which the $P3YSO_3$ moiety then interacts strongly enough to prevent antibody binding (Figures 3G and 3H). Finally, using the direct gp120-coreceptor interaction assay described in Figure 1, we also demonstrated that mCD4-$P3YSO_3$ very potently inhibits gp120 binding to both CCR5 and CXCR4 (Figures 3I and 3J). This suggests that this compound could be a coreceptor independent HIV-1 entry inhibitor.

mCD4 Linked $P3YSO_3$ Peptides Display Strong Antiviral Activity

Having characterized the binding mechanism of these compounds, we investigated whether these anionic peptides, either conjugated or not to mCD4 displayed anti-HIV-1 activity. This was performed using an assay in which viral replication was measured (reverse transcriptase quantification) in the supernatant of blasted peripheral blood mononuclear cells (PBMCs) isolated from three to four donors and infected by either of the HIV-1 reference strains R5 (Ba-L) or X4 (LAI). When used alone, none of the peptides demonstrated antiviral activity at the highest concentration tested (500 nM; data not shown). However, when conjugated to mCD4, they displayed inhibitory activity against the LAI strain, with effective doses giving 50% inhibition (ED_{50}) as low as 0.5 nM for mCD4-$P3YSO_3$, which compares well to 1.4 nM for mCD4-HS_{12}. Consistently with the biochemical data, the importance of the sulfate groups was shown by the large increase of ED_{50} (98 nM) that characterized mCD4-P3Y, whereas the other anionic peptides (mCD4-P3pF, mCD4-P3Asu, and mCD4-E13) displayed 8.2–30 nM ED_{50} (Figure 4A). The Ba-L strain was also very strongly inhibited by mCD4-$P3YSO_3$, with an ED_{50} of 1.3 nM versus 18 nM for mCD4-HS_{12}. None of the other conjugates displayed significant antiviral activity (Figure 4B). AZT, used as a reference anti-HIV molecule in the same assay returned ED_{50} of 8.7 and 11 nM for R5 and X4 viruses, respectively (Figures 4A and 4B).

We also observed that mCD4-$P3YSO_3$ does not need to be preincubated with the virus to be active. Indeed, addition of the molecule either to the cells prior to the viral challenge or to the virus prior to the cell infection return identical results (Table S2). This is consistent with the high affinity this molecule displays for the viral envelope, presumably enabling a fast binding to its target, and also suggests a potential use of this kind of compounds as a microbicide, a condition in which inhibitors are present within the host tissues, before viral infection.

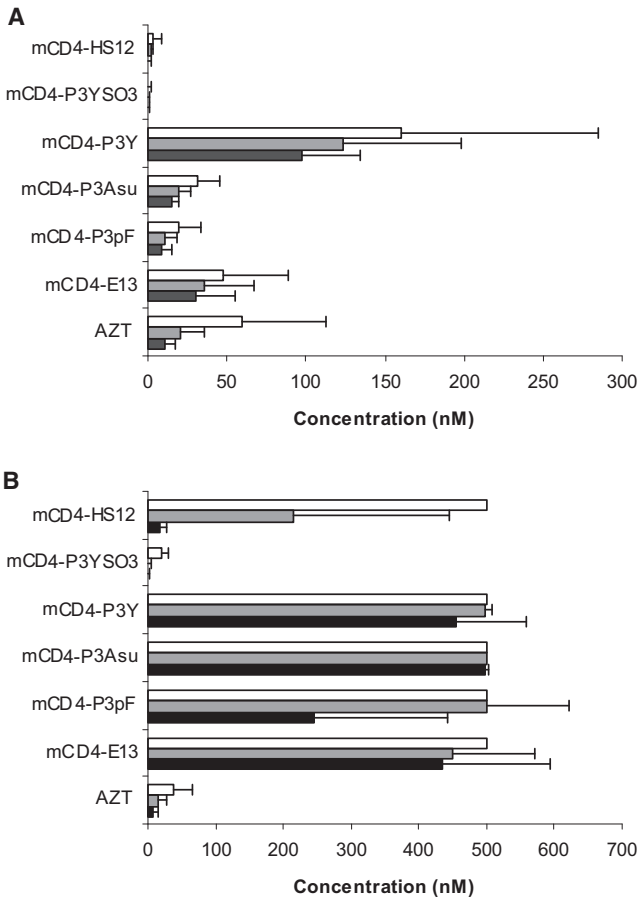


Figure 4. Antiviral Activity of mCD4 Linked to Either HS_{12} or $S(XDXS)_3$ HS Mimetic Peptides

PHA-P-activated PBMCs were infected with either (A) LAI (X4 tropic) or (B) Ba-L (R5 tropic) HIV-1 strains, preincubated with each of the drugs under investigation (1:5 dilutions between 500 nM and 320 pM). Molecules and viruses were maintained throughout the culture, and cell supernatants were collected at day 7 postinfection. Reverse transcriptase activity was quantified from which 50 (black), 70 (gray), and 90% (white) effective doses (ED) were calculated. In the absence of the inhibitory compounds, the RT level was in the range of 10,000–25,000 and 6,500–10,000 pg/ml (depending on the donor) for LAI and Ba-L strains respectively. Data are represented as mean of triplicate experiments (±SEM) performed on PBMCs from three to four donors. See also Table S2.

Having established that mCD4-$P3YSO_3$ displayed very strong antiviral activity against LAI and Ba-L HIV-1 strains, used as model systems, we extended our investigations to using a series of more clinically relevant primary strains, including 92UG029, SF162, 92US723, 96USHIPS4, 92HT599, and 98IN017. As shown in Table 1, mCD4-$P3YSO_3$ displayed a high level of antiviral activity, characterized by ED_{50} in the range of 0.2–1.2 nM for five of them and 29 nM for HIV-1 98IN017. As for the LAI and Ba-L strains, the mCD4 or $P3YSO_3$ were only poorly active or inactive, further supporting the very strong synergistic effect induced by the coupling strategy. None of the molecules showed cytotoxicity at up to 1 μM (data not shown).

Table 1. Anti-HIV-1 activity of AZT, mCD4-P3YSO$_3$, P3YSO$_3$, and mCD4 against Clinical HIV-1 Isolates

Viral Strain Clade-Tropism		92UG029 A-X4	SF162 B-R5	92US723 B-R5/X4	96USHIPS4 B-R5/X4	92HT599 B-X4	98IN017 C-X4
AZT	ED$_{50}$	7 ± 0	8 ± 7	8 ± 0.1	19 ± 9	9 ± 4	8 ± 3
	ED$_{70}$	16 ± 3	13 ± 8	17 ± 1	27 ± 11	22 ± 5	19 ± 5
	ED$_{90}$	61 ± 17	31 ± 3	59 ± 19	56 ± 15	110 ± 13	108 ± 25
mCD4-P3YSO$_3$	ED$_{50}$	0.2 ± 0.0	0.3 ± 0.2	0.3 ± 0.1	1.2 ± 1	0.5 ± 0.2	29 ± 18
	ED$_{70}$	0.3 ± 0.1	0.4 ± 0.3	0.35 ± 0.2	1.6 ± 1.2	1.3 ± 0.9	147 ± 9
	ED$_{90}$	0.8 ± 0.3	0.9 ± 0.2	0.45 ± 0.2	3 ± 1.4	3.5 ± 0.0	>500
P3YSO$_3$	ED$_{50}$	>500	>500	>500	>500	>500	>500
	ED$_{70}$	>500	>500	>500	>500	>500	>500
	ED$_{90}$	>500	>500	>500	>500	>500	>500
mCD4	ED$_{50}$	403 ± 76	245 ± 155	23 ± 1	>500	355 ± 155	>500
	ED$_{70}$	>500	352 ± 105	34 ± 10	>500	>500	>500
	ED$_{90}$	>500	>500	52 ± 22	>500	>500	>500

The table shows the effective dose (ED, mean of triplicate determination), in nM (±SD) required to inhibit 50%, 70%, and 90% of HIV-1 replication.

DISCUSSION

Targeting gp120 for HIV-1 inhibition is both attractive (because the protein engages multiple interactions key to viral entry, thus offering multiple sites for inhibition) and challenging (in the entry complex, the buried surface to block comprises both the gp120-CD4 and gp120-coreceptor interfaces). Although protein-protein interfaces are often relatively featureless and devoid of traditional cavities into which a small molecule can dock, the realization that the gp120 coreceptor binding site displays a restricted number of functionally important basic residues has very recently attracted the attention of many studies. Many of them reported that anionic molecules target the CD4i epitope, as shown by their ability to competitively inhibit mAb 17b binding with IC$_{50}$ in the 1–100 μM range (Acharya et al., 2011; Brower et al., 2009; Cohen et al., 2008; Cormier et al., 2000; Crublet et al., 2008; Dervillez et al., 2010; Farzan et al., 2000; Kwong et al., 2011; Seitz et al., 2010). HS belongs to this class of CD4i domain targeting molecules (Crublet et al., 2008), and a highly sulfated and regular sequence comprising 12 monosaccharide units has been recently prepared. Conjugated to mCD4, it displays strong anti-HIV-1 activity (Baleux et al., 2009). However, HS is extraordinary complex and heterogeneous in sequence (Esko and Lindahl, 2001). Based on the 48 different units that the polymer theoretically comprises, a 12 mer library would reach 10^{10} molecules. Although the reality is less (all the combinations are not possible), it remains much more than can be realistically synthesized for structure-activity relationship studies. Thus, to further develop this kind of molecule we attempted to design HS mimetic peptides, with the general sequence S(XDXS)$_3$ and showed that, when X was a sulfotyrosine, it binds to the CD4i epitope, blocking mAb 17b with IC$_{50}$ of 3 μM, thus comparing very well with the above-mentioned molecules. Interestingly, this peptide interacts equally well with R5 and X4 gp120, whereas HS especially binds to the X4 envelope (Figures 3E and 3F). More importantly, the conjugation of this peptide to mCD4 dramatically enhances its binding activity, the conjugated molecule being able to fully prevent the gp120/mAb 17b interaction at low nM concentration, showing that the covalent linkage induced a strong synergistic effect. This is consistent with the view that high-affinity mCD4 binding takes place initially, inducing the exposure of the mAb 17b epitope to which the sulfated peptide can then bind. As such, this molecule is distinct from other mAb 17b blocking peptides that suppress CD4 binding and subsequent coreceptor binding site exposure through an allosteric inhibitory effect rather than competitive inhibition (Biorn et al., 2004).

Although widely used as a CCR5 or CXCR4 surrogate, mAb 17b, however, only imperfectly defines the gp120 coreceptor binding site which, in addition to the CD4 induced bridging sheet, is also constituted by the V3 loop in particular (Dragic, 2001). Thus, to better assess the blocking efficiency of molecules targeting the gp120-coreceptor interaction, and taking into account domains outside the CD4i epitope itself, CCR5 and CXCR4 were solubilized and functionally captured on top of biacore sensorchips. Binding of gp120 to CCR5 and CXCR4 proved to be both CD4 and concentration dependent and inhibited by specific antagonists. Fitting of the binding data was expectedly complicated by several parameters, such as the complexity of the buffer system used, the reversible nature of both the 1D4-coreceptor and mCD4-gp120 complexes and the conformational flexibility of gp120, thus the calculated affinity values reported should probably be considered as estimates only. Nevertheless, we report K$_D$s of 10 and 150 nM for the YU2-CCR5 and MN-CXCR4 interactions respectively, comparable to those obtained with cellular systems in which the coreceptors remained in their natural cell membrane environment (Babcock et al., 2001; Doranz et al., 1999). This assay provides a useful, label-free method, to identify both binding capacity of envelopes and inhibitory activity of potential drugs. This was especially true in the framework of this study investigating sulfated/polyanionic compounds to target the gp120 coreceptor binding site. Although tyrosine sulfation of coreceptors has been shown to play a less significant role in CXCR4- than in CCR5-dependent HIV-1 entry (Farzan et al., 2002), we found that when conjugated to mCD4 the sulfated P3YSO$_3$ displays very strong binding activity toward both R5- and X4- gp120. Using this assay, we indeed report that gp120 binding to both CCR5 and CXCR4

was fully inhibited by 1:1 stoichiometric condition of mCD4-P3YSO$_3$. The overall positive charge of the V3 loop, which is much higher in X4- than in R5-gp120 (Moulard et al., 2000) strongly influences the electrostatic potential of the coreceptor binding region of the protein. In the case of CXCR4-using viruses, electrostatic interactions between the sulfated peptide and the V3 loop may thus also participate in the blocking mechanism. This view is consistent with the fact that the V3 loop (which importantly contributes to coreceptor binding) is located close to the CD4i bridging sheet and with its known capacity to interact with polyanions (Moulard et al., 2000). This is further supported by the observation that all the anionic peptides prepared during the course of this study (mCD4-P3Asu, mCD4-P3pF, mCD4-E13, and mCD4-P3Y) also display some level of antiviral activity against X4- but not against R5- viruses. This also suggests that, in engineering such compounds, it should be advantageous to use sulfated peptides with only modest specificity so that they can broadly target distinct envelopes, the high specificity of the conjugated bivalent compound being brought by the mCD4 moiety. Structural studies of mCD4-P3YSO$_3$, in complex with different gp120 would be interesting approaches to further define these aspects. In this regard, it can be noted that sulfated peptides would represent an advantage over HS, the crystallography of which, in complex with proteins appearing to be specially challenging (Imberty et al., 2007).

Although relatively limited in molecular mass (5,500 Da) the mCD4-P3YSO$_3$ molecule has the remarkable property to target two critical and conserved regions of gp120, and thus to simultaneously block two large protein surfaces (i.e., the CD4 and the coreceptor binding site). In complete agreement with the biochemical data, it displays 1 nM ED$_{50}$ anti-HIV-1 activity, for both CXCR4 and CCR5 using model viruses in a cellular assay. Importantly, we also found that this compound had a broad neutralizing activity and was very effective against a number of HIV-1 clinical isolates, strongly suggesting that this approach deserves further investigation toward in vivo evaluation. No effective antagonistic inhibitors yet exist for CXCR4. This compound, which at 1 μM is devoid of toxicity, could be a valuable weapon against the more aggressive CXCR4-tropic HIV-1 strains or for patients featuring a mixed HIV-1 population for which CCR5 antagonist cannot be used.

SIGNIFICANCE

While very significant progress has been made in the development of anti-HIV-1 drugs, the emergence of drug-resistant viruses, the inability of current therapy to be curative, and its adverse side effects have led to an urgent need for new blocking strategies. As a target, gp120 that features the coreceptor binding site is particularly attractive. However, its cryptic nature makes it a difficult target that up to now has resisted attacks.

Here, we covalently linked a sulfotyrosine containing tridecapeptide that targets the gp120 coreceptor binding site, to a CD4 mimetic (mCD4). We showed that the mCD4, in interacting with gp120, induces conformational changes that expose the coreceptor binding site and renders it available to be blocked by the sulfated peptide. In cellular assays, this compound, which successfully targets two critical domains of gp120, displays strong antiviral activities and neutralizes HIV-1 with 1 nM IC$_{50}$.

The conjugate was much more effective than a mixture of mCD4 and tridecapeptide alone, indicating that the covalent linkage is essential to produce a synergistic effect. To our knowledge, this compound establishes a new type of inhibitor and suggests a concept by which a relatively low specific molecule (the sulfated peptide), coupled to a highly specific compound (the mCD4) can reach very high affinities for its target. Combining these two characteristics may enable the molecule to accommodate mutations that invariably characterize acquired viral resistance.

These results should have strong implications for the development of a new class of anti-HIV-1 therapy: the mCD4-conjugate simultaneously blocks the attachment and entry domains of gp120 and thus inhibits viral replication at a very early stage of the viral life cycle. Most importantly, it has the remarkable and unique property to neutralize both CCR5- and CXCR4-tropic HIV-1. This is definitively a strong advantage since HIV-1 may escape from CCR5 antagonists through selection of CXCR4-using variants.

EXPERIMENTAL PROCEDURES

Materials

A BIAcore 3000 machine, CM4 sensorchip, amine coupling kit and HBS-P (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P20 [pH 7.4]) were from GE-Healthcare. Streptavidin and Piperidin were from Sigma. MN and YU2 gp120 were from Immunodiagnostic. Soluble CD4, mAb 17b and Cf2Th coreceptor expressing cells were obtained through the NIH AIDS Research and Reference Reagent Program. The antibodies 12G5 and 2D7 were purchased from R&D systems and BD Pharmingen, respectively. The HIV-1 entry inhibitors AMD3100 and Maraviroc were from Fernando Arenzana (Pasteur Institute, Paris). The1D4 antibody was from Flint Box, University of British Columbia. Synthetic phospholipid blend 1,2-dioleoyl-sn-glycero-3-phosphocholine/1,2-dioleoyl-sn-glycero-3-phospho-L-serine formulation (DOPC/DOPS; 7:3, w/w), the Mini-Extruder kit, filter supports and polycarbonate filters with defined pore diameter (100 nm) were purchased from Avanti Polar Lipids. Detergents, n-dodecyl-β-D-maltopyranoside (DOM), 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane sulfonate/N,N-Dimethyl-3-sulfo-N-[3-[[3α,5β,7α,12α)-3,7,12-trihydroxy-24-oxocholan-24-yl] amino]propyl]-1-propanaminium (Chaps) and Cholesteryl hemisuccinate tris salt (CHS) were purchased from Anatrace. Complete, EDTA-free protease inhibitor tablets were from Roche Diagnostics. Polyethylene glycol 8,000 50% w/v solution was purchased from Hampton research. Resins for peptide synthesis were purchased from RAPP Polymere GmbH and Fmoc AAs, HATU, NMP, DMF, and TFA were from Applied Biosystems. Fmoc-Tyr (SO3.NnBu4)-OH and Fmoc-γ-Aminobutyric-OH (γ-Abu) were from Novabiochem, (S)-Fmoc-2-amino-octanedioc acid-8-ter-butyl ester (Asu) from Polypeptides, and Fmoc -L-4 (O-tButylcarboxymethyl)-Phe-OH (pF) from Anaspec. HPLC grade triethylamine acetate buffer was from GlenResearch. *N*-succinimidyl-S-acetylthiopropionate (SATP) was from Pierce.

CCR5/CXCR4 Solubilization

The human receptors CCR5 and CXCR4, featuring a C-terminal C9 tag (TETSQVAPA), were expressed in Cf2Th canine thymocyte cells as described previously (Mirzabekov et al., 1999). The CCR5 and CXCR4 solubilization protocol was adapted from a described procedure (Navratilova et al., 2005). Briefly Cf2Th.CCR5- or CXCR4-expressing cells (5–8 × 10^6) were solubilized in 1 ml buffer consisting of 100 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 7.5), 10% glycerol, 15% PEG 8000, protease inhibitors, CHS (0.2%), DOM (1.5%), CHAPS (1.5%), and 0.33 mM DOPC:DOPS liposomes (see detailed buffer preparation in the Supplemental Experimental Procedures). The cell suspension was sonicated (6 × 1 s pulses) and placed on a rotating wheel

at 4°C for 3 hr. The solutions containing the solubilized coreceptors were centrifuged at 14,000 rpm for 30 min at 4°C and the supernatants were either used directly in SPR analysis or stored at −80°C until further use.

Surface Plasmon Resonance-Based Binding Platform

The interactions between gp120 and its ligands (CD4, mAb 17b, CCR5, and CXCR4) were analyzed by surface plasmon resonance (SPR) technology. For that purpose, N-ethyl-N′-(diethylaminopropyl)-carbodiimide (EDC)/N-hydroxy-succimide (NHS) activated CM4 sensorchips were functionalized with either 1,200 RU of soluble CD4, 700 RU of mAb 17b, or 7,000 RU of mAb 1D4 and blocked with pH 8.5 1 M ethanolamine. The C9-tagged CCR5 or CXCR4 were captured onto the 1D4 mAb to a level of ~4,000 RU. In some cases, gp120 were also immobilized onto CM4 sensorchip. For this, MN (50 μg/ml in 5 mM maleate buffer [pH 6]) or YU2 (50 μg/ml in 10 mM acetate buffer [pH 4.8]) were injected at 5 μl/min over an EDC/NHS activated flow cell until levels of 4,500 RU was obtained. Molecules under investigation were injected over the different surfaces and the binding responses were recorded as a function of time (see Supplemental Experimental Procedures).

Peptide Synthesis and Purification

Peptides were prepared by solid-phase peptide synthesis on H-Ser(tBu)-2-ClTrt-PS-resin using Fmoc chemistry excepted for the E13 peptide which was prepared on Fmoc-Glu(tBu)-PHB-PS-resin. Fmoc-Tyr-(SO3.NnBu4)-OH was used to synthesize the sulfotyrosines containing peptide. SATP was used to introduce a protected sulfhydryl groups at the N terminus of each purified peptide, which were then conjugated in presence of hydroxylamine to a K^5 maleimide-activated mCD4, the synthesis of which has been reported elsewhere (Baleux et al., 2009) to yield the desired conjugates mCD4-P3YSO_3, mCD4-P3Y, mCD4-P3pF, mCD4-P3Asu and mCD4-E13. All compounds were purified by RP-HPLC. Analytical procedures, characterization, and quantification of these materials are described in the Supplemental Information.

Antiviral Assay

Phytohemagglutinin (PHA)-P-activated PBMCs were infected either with the reference lymphotropic HIV-1/LAI strain (Barré-Sinoussi et al., 1983) or with the reference macrophage-tropic HIV-1/Ba-L strain (Gartner et al., 1986). These viruses were amplified in vitro with PHA-P-activated PBMCs, and 50% tissue culture infectious doses ($TCID_{50}$) were calculated using Kärber's formula (Kärber, 1931). Viruses (125 $TCID_{50}$) were incubated for 30 min with five concentrations (1:5 dilutions between 500 nM and 320 pM) of each of the molecules to be tested and added to 150,000 PBMCs (moi ~0.001). Cell supernatants were collected at day 7 postinfection and stored at −20°C. In some cases, the compounds were added to the cells prior to viral challenge. Viral replication was measured by quantifying reverse transcriptase (RT) activity in the cell culture supernatants using the Lenti RT Activity Kit (Cavisi) and AZT was used as reference anti-HIV-1 molecule. In parallel, cytotoxicity was evaluated on day 7 in uninfected PHA-P-activated PBMC using a colorimetric methyl-tetrazolium salt (MTS/PMS) assay (Promega). Experiments were performed in triplicate and 50, 70 and 90% effective doses (ED) were calculated using SoftMaxPro software.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.chembiol.2011.12.009.

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